Nitric oxide (NO) exerts physiological effects by activating specialized receptors that are coupled to guanylyl cyclase activity, resulting in cGMP synthesis from GTP. Despite its widespread importance as a signal transduction pathway, the way it operates is still understood only in descriptive terms. The present work aimed to elucidate a formal mechanism for NO receptor activation and its modulation by GTP, ATP, and allosteric agents, such as YC-1 and BAY 41-2272. The model comprised a module in which NO, the nucleotides, and allosteric agents bind and the protein undergoes a conformational change, dovetailing with a catalytic module where GTP is converted to cGMP and pyrophosphate. Experiments on NO-activated guanylyl cyclase purified from bovine lung allowed values for all of the binding and isomerization constants to be derived. The catalytic module was a modified version of one describing the kinetics of adenylyl cyclase. The resulting enzyme-linked receptor mechanism faithfully reproduces all of the main functional properties of NO-activated guanylyl cyclase reported to date and provides a thermodynamically sound interpretation of those properties. With appropriate modification, it also replicates activation by carbon monoxide and the remarkable enhancement of that activity brought about by the allosteric agents. In addition, the mechanism enhances understanding of the behavior of the receptor in a cellular setting.

Nitric oxide (NO) is normally generated mainly by endothelial and nerve cells and subserves an intercellular messenger role in most tissues, producing diverse effects, such as smooth muscle relaxation, inhibition of platelet aggregation, and synaptic plasticity (1, 2). Physiological NO signals are transduced through receptors equipped with guanylyl cyclase (GC) activity, leading to the intracellular formation of the effector molecule, cGMP (3). The discovery in homogenates of a “soluble” GC (4) that could be activated by NO (5) was vital for the identification of NO as an endogenous signaling agent. Nevertheless, despite many years of study, the mechanism of NO signal transduction through this pathway and the ways it is regulated remain indistinct. This is disappointing, because, as exemplified by synaptic transmission (6, 7), a quantitative understanding of how cells decode incoming signals provides powerful insight into the language of chemical communication and helps pinpoint abnormalities in disease states.

In the current scheme (Fig. 1), the NO binding site is a heme prosthetic group, the occupation of which results in disengagement of a coordinating histidine bond, which helps drive a conformational change that propagates to the catalytic domain, resulting in the conversion of GTP into cGMP and pyrophosphate. Armed with spectroscopic data on the rates of binding and dissociation of NO (8–10) and with functional data on the potency and efficacy of NO and the rates at which cGMP synthesis activates and deactivates (11–18), it has been possible to insert values for the rates of the binding and isomerization steps depicted in the basic scheme (Fig. 1) and arrive at a reasonable approximation of the behavior of the NO-activated enzyme (19–21).

Although a good starting point, the two-step reaction ignores many other regulatory mechanisms. As well as being a substrate, GTP has been pictured as facilitating the NO-induced conformational change in a manner that is inhibited competitively by physiological concentrations of ATP (22, 23). An uncompetitive component to the activity of ATP has also been identified (23), depicted in Fig. 1 as being at the catalytic site. There are, in addition, sites where allosteric modulators, such as YC-1, act to augment NO-evoked activity (24) putatively by inhibiting the rate at which the enzymatically active species reverts to its inactive state (20). Although giving a reasonable empirical description, the resulting phenomenological scheme (Fig. 1) gives little useful information about the underlying mechanisms. In reality, the protein is an enzyme-linked receptor having binding sites for various agonists, antagonists, and modulators and an integral catalytic domain, where substrate and products bind and unbind. We sought here to gather the data necessary for assembling a more complete, mechanism-based model of the receptor-enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—2–4-Carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), diethylamine NONOate, spermine NONOate, purified bovine lung GC (soluble guanylyl cyclase), and BAY 41-2272 were obtained from Axoxa (UK) Ltd. (Nottingham, UK). Remaining special chemicals were from Sigma. NONOate stock solutions were made up in 10 mM NaOH on the day of use and kept on ice.
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**Measurement of GC Activity**—The purification of the bovine lung protein used and its characteristics have been described in detail (25). As before (26), the supplied protein (250 μg/ml in 50% glycerol, 88 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, 25 mM triethanolamine at pH 7.4) was diluted to 5 μg/ml in a buffer containing 50 mM Tris, 1 mM dithiothreitol, and 0.5% bovine serum albumin and maintained on ice; aliquots were diluted 1:100 in assay buffer (50 mM Tris, 100 μM EDTA, 0.05% bovine serum albumin, 0.3 mM urate, 1000 units/ml superoxide dismutase, pH 7.4, at 37 °C) containing GTP (0.01–1 mM), ATP (0–10 mM), and MgCl₂ (0.1–1.5 mM), prewarmed to 37 °C. NO was usually delivered in concentrations giving 300 μM NO in a buffer containing 50 mM Tris, 1 mM dithiothreitol, and 0.5% bovine serum albumin at pH 7.4, and MgCl₂ in concentrations giving 300 μM NO in excess of the total nucleotide concentration, prewarmed to 37 °C. NO was usually delivered in known, clamped concentrations by adding combinations of the NO donor spermine NONOate and the NO scavenger CPTIO as described (13). In some experiments (specified under “Results”), a saturating NO concentration was applied using diethylenetriamine NONOate (1 μM). The incubations were at 37 °C and, unless stated otherwise, lasted for 2 min, after which aliquots were removed and inactivated in boiling 50 mM Tris, 4 mM EDTA buffer (pH 7.4), and cGMP was subsequently measured by radioimmunoassay. In each experiment, three or four independent runs were carried out for each condition, and the resulting data are presented as means ± S.E.

**Model Formulation**—The model comprised a binding module with a juxtaposed catalytic site. The full binding module (Fig. 2A) is analogous to the cubic ternary complex used for modeling G-protein-coupled receptors (27). The very low activity observed in the absence of NO (see “Results”) allows the binding scheme to be simplified, as shown in Fig. 2B. Catalysis proceeds from the active, NO- and GTP-bound species and is assumed to follow the same mechanism deduced for adenyl cyclase (28), wherein GTP is first split into cGMP and pyrophosphate, and then the products leave one at a time (Fig. 2B).

The binding steps are likely to be close to equilibrium when activity in the absence of NO (see “Results”) allows the reaction as a whole is at steady state, so the first aim was to obtain estimates of the equilibrium constants. Using the notation of Colquhoun (29) for the cubic ternary complex (Fig. 2A), when activity in the absence of agonist is low (i.e. when the value of $E_A$ in Fig. 2A is small), the EC₅₀ for NO is given by Equation 1,

$$\text{EC}_{50}(\text{NO}) = \frac{K_A (1 + \frac{G}{K_{GA}})}{1 + \frac{G}{K_{GA}} + E_A (1 + \frac{G}{K_{GA'}})} \quad \text{(Eq. 1)}$$

where $G$ represents the GTP concentration, and other parameters are as in Fig. 2A. This equation may also be written as follows.

$$\text{EC}_{50}(\text{NO}) = \frac{K_A (1 + \frac{G}{K_{GA}})}{1 + \frac{G}{K_{GA}} + E_A (1 + \frac{G}{K_{GA'}})} \quad \text{(Eq. 2)}$$

Abbreviating [NO] as $N$, the EC₅₀ for GTP is as follows.

$$\text{EC}_{50}(\text{GTP}) = \frac{K_G (1 + N)}{1 + N + E_g (1 + \frac{N}{K_{GA}})} \quad \text{(Eq. 3)}$$

When [NO] is high relative to its binding affinities, and since $E_A^r/K_{GA} = E_A^r/K_{GA'} = E_A^r/K_{GA} = g$, Equation 3 closely approximates the following.

$$\text{EC}_{50}(\text{GTP}) = \frac{K_G (1 + E_g)}{g(1 + E_g)} \quad \text{(Eq. 4)}$$

Since $E_{AG} = K_{GA} E_A^r K_{GA}$, Equation 4 may also be written as follows.

$$\text{EC}_{50}(\text{GTP}) = \frac{K_G K_{GA} (1 + E_g)}{E_A^r K_{GA} + g K_{GA'}} \quad \text{(Eq. 5)}$$

By finding the EC₅₀(NO) at two different concentrations of GTP, equating them using Equation 2 (whence $K_A$ cancels out), solving for $g$, and inserting the result in Equation 5, $E_A$ and $E_{AG}$ cancel out, and the value of $K_G$ comes to the following.

$$K_G = \frac{GEI (f - r) + G^2 r (f - 1)}{E (1 - f) + G (1 - fr)} \quad \text{(Eq. 6)}$$

where $E$ represents the EC₅₀(GTP) at high [NO], and $f$ is the factor by which the EC₅₀(NO) increases when [GTP] (G) is reduced by the factor $r$. In the results, we see that with [GTP] = 1 mM, $f = 1.57$ when $r = 0.1$, and the EC₅₀(GTP) at 50 mM NO is 64 μM, giving $K_G = 187 \mu M$. Having this value and a value of $K_{GA}$ restricts the values of all of the other equilibrium constants. For example, putting $K_G = 187 \mu M$ and $K_{GA} = 25 \mu M$ (see “Results”) into Equation 5 gives the relationship between EC₅₀(GTP), $g$, and $E_A$. With an EC₅₀(GTP) of 64 μM, $g = 2.92 - 4.56 E_A$. Since $g$ must be at least 1 (otherwise NO would become less potent as [GTP] increases, contrary to observation), the maximal value of $E_A$ (when $g = 1$) comes to 0.42, and the maximal value of $g$ (when $E_A = 0$) is 2.92. Estimates of the ranges of NO binding affinities ($K_A$ and $K_{GA}$) are arrived at from Equation 2 using minimum and maximum values of $E_A$ and the corresponding values of $g$ (Table 1).

A further constraint comes from measuring the increase in maximal GC activity observed in the presence of an allosteric enhancer (see “Results”); assuming this enhanced response is maximal, the efficacy normally is about 70% of maximum. The main determinant of efficacy is the constant governing the transition of the NO- and GTP-bound species to its active form (E_{AG} in Fig. 2); setting $E_{AG} = 1$ gives the desired efficacies in the absence and presence of the allosteric enhancer (when the full
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Model is run and also further restricts the value of the constant for the corresponding GTP-free state ($E_A$; Table 1). Even allowing for errors in the estimation of the EC$_{50}$ (GTP), the range of suitable constants becomes very limited (Table 1). By methodically altering the value of the factor $g$, the missing equilibrium constants best describing the data in Fig. 4 can be easily found; $g = 2.0$, $K_A = 3.65$ nM, $K_{AG} = 1.83$ nM, $K_{GA} = 94$ µM, and $E_A = 0.27$ give EC$_{50}$ (NO) values of 1.02 and 1.65 nM in the presence of 1 and 0.1 mM GTP, respectively, and an EC$_{50}$ (GTP) of 62 µM in the presence of 50 nM NO.

Conversion of GTP into cGMP and pyrophosphate is assumed to follow the corresponding reaction in adenylyl cyclase because of the very similar underlying chemistry and the close homology of the catalytic centers, to the extent that swapping three amino acids is sufficient to convert NO-stimulated GC into NO-stimulated adenylyl cyclase (30). The kinetics of a soluble form of adenylyl cyclase have been described (28) and formed the basis of the GC catalytic module (Fig. 2B).

A few adjustments were needed to improve thermodynamic balance and for the module to comply with data on GC. First, the maximum activity was raised to 15 µmol/mg/min, which was the typical value found in the present experiments, by increasing the constants for the rate-limiting steps (NR*G to NR* and NR* to NR*). Second, the rate constants for product binding were modified to give the inhibition by cGMP and pyrophosphate measured using recombinant NO-activated GC (31). It was necessary to include additional states that bind pyrophosphate to comply with the data (see “Results”); inclusion of these states made no significant difference to the other aspects of the model, so they were omitted for clarity elsewhere. Finally, the product binding and unbinding cycle (the upper right-hand triangle in Fig. 2B) was made to conform to the thermodynamic principle of microscopic reversibility (32). This was achieved by increasing the rate constants for the minor product dissociation pathway (NR* to NR* and then to NR*) by factors of 10, a modification that had little effect on the output of the model.

Using the deduced equilibrium constants for the binding module, introduction of catalysis caused a slight mismatch to the data (e.g. the modeled EC$_{50}$ (NO) values in the presence of 1 and 0.1 mM GTP fell to 0.67 and 1.15 nM from 1.02 and 1.65 nM, respectively). This arises because the draining of GTP at the catalytic site pulls the equilibrium in that direction. Changing the value of the factor $g$ from 2.0 to 1.5 and setting the NO binding affinity for the GTP-free species ($K_A$) to 4.23 nM (hence, altering $E_A$ to 0.2) gave the values that enabled good fits to all of the data reported here. To interface with the catalytic module, the equilibrium constants for the binding module had to be converted into microscopic rate constants. The bimolecular association rate for NO ($3 \times 10^6$ M$^{-1}$ s$^{-1}$) was assigned from spectroscopic experiments, yielding $>1 \times 10^7$ M$^{-1}$ s$^{-1}$ at 15 °C (9) and $>1.4 \times 10^7$ M$^{-1}$ s$^{-1}$ at 4 °C (10). NO dissociation rates were the dissociation constants given in Fig. 2B multiplied by the association rate. The bimolecular association rate for GTP binding was initially set at $1 \times 10^6$ M$^{-1}$ s$^{-1}$ with dissociation rates then being set by the equilibrium constants. Tests for reactions at equilibrium were made by altering the forward and backward rate constants over a wide range (100-1000-fold, keeping the ratio the same) under conditions of varied NO and GTP concentrations, a discrepancy of 10% in cGMP output between the two extremes signifying a nonequilibrium state. All NO binding steps and most GTP binding steps were effectively at equilibrium. The reactions out of equilibrium were, in order of degree, those represented by the constants $K_{GA}$, $E_{AG}$, and $E_A$ in Fig. 2. This arises because these reactions are closest to the catalytic site where GTP is consumed. Assignment of microscopic rate constants for these steps was on the basis of fits to experimental data (see supplemental Fig. S1). For ATP, all of the binding steps were effectively at equilibrium under steady-state conditions; the bimolecular rate constants for ATP binding to the GTP-free and GTP-bound states were set at $5 \times 10^5$ and $3 \times 10^6$ M$^{-1}$ s$^{-1}$, respectively.

For allosteric modulation, from the model presented under “Results” (see Fig. 8), the EC$_{50}$ for the allosteric enhancer (Y) under conditions of high [NO] and [GTP] and when $E_{AG}$ is $>1$ approximates the following,

$$EC_{50}(Y) = \frac{K_Y(1 + E_{AG})}{E_{AG}} \quad \text{(Eq. 7)}$$

where $E_{AG} = 1$ (see above) and $E_{AGY} = K_Y/K_{Y^*}$, giving $K_{Y^*} = EC_{50}(Y)/2$. To complete the binding module, a starting value for $E_{AGY}$ was obtained from the rate of deactivation under conditions of high [NO] and [GTP], which is explicitly dependent on the microscopic rate constants for this step as well as on the rate of NO unbinding (19). Keeping the forward rate constant unaltered (100 s$^{-1}$), the deactivation rate constant (about $8 \times 10^{-4}$ s$^{-1}$; see “Results”) suggests a value for the backward rate constant of about 0.1 s$^{-1}$. From this value, $E_{AGY} = 1000$ and $K_Y$ is 1000 times the value of $K_{Y^*}$ (found as above). Fine adjustments were made to obtain the best fits to the data. Microscopic rates for the unbinding of Y were calculated from the equilibrium constants, assuming a bimolecular association rate of $1 \times 10^6$ M$^{-1}$ s$^{-1}$.

Differential equations drawn up to describe the steps in the model were solved using Mathematica 11 or 14 (Adept Scientific, Letchworth, UK). The model output is shown in many of the illustrations in the form of broken lines; conversion of model data (the rate of cGMP formation being in units of s$^{-1}$) into the experimental units (pmol of cGMP formed/mg/min) assumed a molecular mass of 150 kDa for the NO receptor–enzyme. To facilitate comparison with experimental results, the model data are normalized to the measured activity, which varied from day to day presumably as a result of differences in the amount of active protein.

RESULTS

In aiming to formulate a mechanism for NO-stimulated GC activity, the starting point was with the binding of NO and GTP. It was assumed that there is a single NO binding site, because NO concentration-response curves are monophasic with a slope of 1.0 (13, 33). For the same reasons, it was also assumed that there is a single site for GTP binding, which must be at the catalytic site. To be thermodynamically complete, there are eight different states, represented as a cube in Fig. 2A. This representation is analogous to the “cubic ternary complex” used.
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FIGURE 2. Enzyme-linked receptor model for NO-activated GC. A, cubic representation of the full NO receptor binding module. R, receptor protein. Some of the mathematical interrelations are given in parentheses. g is a constant describing the increased affinity for GTP when NO is bound and, reciprocally, the increased affinity for NO when GTP is bound; h is the constant describing the increased tendency for a conformational change when NO is bound and the corresponding increase in affinity for GTP in the active (asterisk) relative to the inactive NO-bound receptor. B, reduced model (ignoring the unliganded active species in A) together with the final values of the equilibrium constants or microscopic rate constants (m/s or s).

to model G-protein-coupled receptors, where G in Fig. 2A (here standing for GTP) represents the G-protein (27). The module allows for all possible interactions with NO at the ligand binding site and for GTP at the catalytic site and assumes that the interactions are governed by the laws of mass action. It further assumes that the receptor is either inactive or active, the latter states being represented by asterisks in Fig. 2A. Catalysis proceeds from the active GTP-bound states (R*G and NR*G), forming cGMP and pyrophosphate, which then dissociate, either one first followed by the other (as in Fig. 2B).

Basal Activity—In the full version (Fig. 2A), catalysis could occur in the absence of NO (from the R*G species); this would represent a basal GC activity analogous to the intrinsic activity of some G-protein-coupled receptors (34). Usually, basal GC activity is taken to be that measured in the absence of added NO, but environmental NO will always be present in amounts that change with geographical location and time of day (35). The unstimulated activity we recorded varied appreciably, ranging from 50 to 1500 nmol/mg/min, consistent with the fluctuations in environmental NO recorded in the locality (supplemental Fig. S2). To determine the true basal activity, the rate of cGMP formation was measured with increasing concentrations of the NO scavenger, CPTIO (Fig. 3A). On a day when the "basal" activity was fortuitously high (1.5 μmol/mg/min), CPTIO caused a concentration-dependent inhibition (IC50 = 2.3 μM), the activity remaining at the highest CPTIO concentration tested (3 mM) being very low (3 nmol/mg/min). This effect could not be ascribed to an unexpected inhibitory side effect of CPTIO on the receptor-enzyme, because, when the CPTIO concentration was varied 10-fold while maintaining the NO concentration constant (1 nM), there was no significant effect on the rate of cGMP formation (Fig. 3B). It can be concluded, therefore, that the true basal activity is at least 5000-fold lower than is observed maximally in the presence of NO (about 15 μmol/mg/min); accordingly, catalysis from the active unliganded species (R*G) can be disregarded.

NO and GTP Binding—The very low activity in the absence of NO also means that the corresponding states in the mechanism (Fig. 2A) can be left aside, leading to the simplified model shown in Fig. 2B. The reactions of the binding module are likely to be close to equilibrium when the reaction as a whole is at steady state (36), so the next step was to obtain estimates of the equilibrium constants. The best estimate of the binding constant for GTP in the active catalytic site (Kd1 in Fig. 2B) is obtained under conditions of maximal activity, which is seen in the presence of a saturating concentration of an allosteric enhancer (see below). The observed value was 25 μM. The EC50 for NO is explicitly dependent on the GTP concentration. By measuring this dependence and also the EC50 for GTP at a high NO concentration, the constant for GTP binding to the unliganded receptor (Kd) can be found (see "Experimental Procedures"). Reducing the GTP concentration from 1 mM to 100 μM resulted in only a small rightward shift in the NO concentration-response curve (Fig. 4A), the EC50 increasing from 1.0 to 1.6 nM. This result, together with an EC50 for GTP of 64 μM in the presence of 50 nM NO (Fig. 4B), gives the constant Kd = 187 μM. The ranges of possible values for the remaining constants, especially those governing the binding of NO, were then severely restricted (Table 1). Their final values (Fig. 2B) together with those of the underlying forward and backward rates were assigned as described under "Experimental Procedures.

Catalysis—Catalysis (Fig. 2B) was assumed to follow the same kinetic mechanism described for adenylly cyclase (28), a
mechanism that has been well supported by structural information (37). Adjustments were made to the kinetics to conform better to thermodynamic principles and the known properties of the NO-stimulated GC (see “Experimental Procedures”). The resulting maximum cGMP output is 15 μmol/mg/min (100 mM NO, 3 mM GTP), and it exhibits appropriate product inhibition. The IC_{50} for inhibition by cGMP is 11 mM (100 nM NO, 3 mM GTP), and it is essentially identical to the value reported for recombinant GC (31). Despite being an effective inhibitor of the NO-activated enzyme, cGMP was found to be much weaker than GTP (31). A similar competitive interaction was observed in the presence of 50 nM NO at 100 mM ATP (Fig. 5A). At concentrations up to 3 mM, there was a progressive rightward shift in the inhibition curve as the GTP concentration increased (Fig. 5B), according to a competitive-type effect (23), but, at higher ATP concentrations (5 and 10 mM), the activity fell sharply, indicating an additional mechanism. Since the ATP concentrations producing this additional inhibition are unlikely to be physiological, we have restricted our analysis to 3 mM ATP and below. Traditional enzyme plots showed classical mixed inhibition, the K_{i} at the competitive site being about 370 μM compared with a K_{i} of about 3 mM at the noncompetitive site (supplemental Fig. S5).

In its simplest form, mixed inhibition occurs when an inhibitor binds to an enzyme at a site distinct from that of the substrate, forming inactive complexes with both the substrate-free and substrate-bound species, the two components giving rise to the competitive and uncompetitive kinetics, respectively (42). Hence, it is logical to suggest that ATP binds to the substrate-free NO receptor with a K_{i} of about 370 μM and to the substrate-bound receptor with a K_{i} of about 3 mM (Fig. 6). A lowering of the ATP affinity when GTP is bound is consistent with (42).

## Table 1

| Parameter | EC_{50}(GTP) at 50 nM NO | 55 μM | 64 μM | 75 μM |
|-----------|--------------------------|-------|-------|-------|
| K_{G} (μM) | 170                      | 187   | 209   |       |
| K_{GAT} (μM) | 25                      | 25    | 25    |       |
| g          | 1.3                      | 1.3   | 1.3   | 1.3   |
| K_{GAT} (μM) | 53–170                  | 64–187| 75–209|       |
| K_{GAT} (nM) | 2.9–4.5                 | 2.7–3.8| 2.6–3.4|       |
| E_{AG}     | ≤3.8                     | ≤3.1  | ≤2.7  |       |
| E_{A} (K_{GAT} = 1) | 0.15–0.45 | 0.13–0.39| 0.12–0.33|       |

5 E. J. Halvey and J. Garthwaite, unpublished observation.
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Incorporating ATP binding into the model in an unbiased way leads to a cubic representation in which the affinities of NO; for example, at 100 μM GTP, 1 mM ATP should modestly increase the EC50 for NO from 1.6 to 2.4 nM. Experimental testing (Fig. 7B) indicated a somewhat greater shift to 3.4 nM, alongside the expected reduction in maximal response. Although the difference is marginal, a similar discrepancy was observed with respect to the rate of deactivation of GC activity elicited by protoporphyrin-IX, a nitrosyl heme mimetic, when the natural ligand-binding heme is missing (17, 47). All of these features strongly suggest that they act by allosteric enhancement/agonism and not by directly affecting ligand binding or catalysis. Allosterism has been well studied for other receptors and enzymes (48, 49), and, with the necessary quantitative information, a formal scheme can be drawn up that, in turn, helps understand the normal functioning of the GC-coupled receptors.

Allosteric agonists and enhancers selectively stabilize active forms of receptors. Indeed, the only site of action that would give all of the features noted above is a preferential stabilization of the active species. Sometimes, allosteric agents produce significant effects through an engagement of the active agonist-free receptor (R* and R*G in Fig. 2A). Tests using a saturating concentration of the compound BAY 41-2272 (100 μM), the most potent and selective of this type of compound generally available (18), showed substantial GC activation in the absence of added NO (Fig. 3A). With increasing concentrations of the NO scavenger CPTIO, however, the activity was progressively reduced, implying that it depended on environmental NO, similarly to the “basal” activity recorded normally (see above). The IC50 for CPTIO was about 100-fold higher against the BAY 41-2272-stimulated activity compared with “basal” activity (220 versus 2.3 μM), a result compatible with the much higher potency of NO in the presence of BAY 41-2272 (see below). The apparent lack of any significant NO-independent activity of BAY 41-2272 implies allosteric enhancement rather than allosteric agonism (50), and it allows the simpler form of the model to serve as a scaffold for the allosteric site (Fig. 8).

In broad agreement with prior reports (18, 51, 52), the EC50 of BAY 41-2272 decreased from 130 nM in the absence of added NO to 40 nM in the presence of 50 nM NO (Fig. 9A). From the latter value, the BAY 41-2272 binding constant for the active receptor (K2, in Fig. 8) comes to 20 nM (see “Experimental Procedures”). Measuring the rate of deactivation upon removal of NO allows the BAY 41-2272 binding constant to the inactive receptor (K1) to be estimated (see “Experimental Procedures”). Deactivation in the presence of a saturating BAY 41-2272 concentration (100 μM) and 1 mM GTP was very slow, being incomplete even after 25 min (Fig. 9B). The curve could be fitted to a

the two binding sites being situated close to each other (43). Mixed inhibition can show deviations from linear kinetics (42), which could introduce complications. The time courses of cGMP formation in response to 50 nM NO in the absence and presence of ATP, however, were both linear up to 2 min (Fig. 7A), suggesting that the derived constants are reasonable approximations of the equilibrium dissociation constants.

FIGURE 6. Model for ATP inhibition of NO receptors. A, ATP; R, receptor protein. Equilibrium constants or microscopic rate constants for the binding and isomerization steps are indicated; values for the catalytic module are the same as in Fig. 28.

FIGURE 7. ATP inhibition is linear with time and lowers the potency of NO. A, time courses of the accumulation of cGMP in the presence of 50 nM NO and 1 mM GTP with (circles) or without (squares) 3 mM ATP. B, concentration-response curves to NO at 100 μM GTP in the absence (squares) or presence (circles) of 1 mM ATP. Data are fitted to the Hill equation (solid lines), giving the stated EC50 values; broken lines (mostly hidden) are model predictions.

this modification, fits to both sets of data were 2-fold improved, so this minor adjustment was maintained.

The resulting model faithfully replicates the inhibition by ATP (up to 3 mM) at different GTP concentrations (Fig. 5, broken lines) and the NO concentration-response curves with and without ATP (Fig. 7B, broken lines).
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The allosteric model accurately replicates the BAY 41-2272 concentration-response curve in the presence of 50 nM NO and suggests that the ambient NO concentration (no added NO) during this experiment was near 100 pM (Fig. 9A). The model suggested further testable predictions. First, evidence was presented earlier that, in the absence of BAY 41-2272, reducing the GTP concentration and adding a physiological ATP concentration lowered the potency of NO; a corollary is that the rate of deactivation must be increased, but this is too fast to measure using manual methods. The half-time for deactivation is predicted to be 2.4 s in the presence of 1 mM GTP, 1.5 s with 100 μM GTP, and 0.6 s with 100 μM GTP and 1 mM ATP. In the presence of BAY 41-2272, however, deactivation becomes slowed and easily measured, and, upon combining the two models (Figs. 6 and 8; see below), it is predicted that the changes in deactivation rate upon altering the nucleotide concentrations should be proportionately almost the same as in the absence of BAY 41-2272. In good agreement, the deactivation rate in the presence of BAY 41-2272 was speeded up when GTP was reduced from 1 mM to 100 μM and further speeded by 1 mM ATP (Fig. 9B), the experimentally determined half-times being close to the predicted values.

Another prediction is that an allosteric enhancer should increase the potency of NO. Such an effect has been indicated before (12, 14, 45, 52), but the NO concentrations single exponential, which gave a half-time of 14.6 min (not shown), or to a deactivation function (16), which gave a half-time of 22 min. From these rates, after optimizing fits to the data, \( K_Y \) comes to 11 μM, and the equilibrium constants governing the transition from inactive to active BAY 41-2272-bound receptor \( \left(K_{G\alpha^*} \right) \) in Fig. 8) are 560-fold higher than normal. This means that, in saturating concentrations of NO and BAY 41-2272, essentially all of the receptor is in its active form. The \( E_{50(GTP)} \) for CTP under these conditions, therefore, comes close to the affinity of the active catalytic site \( (K_{G\alpha^*} \) in Fig. 2). Experimental investigation showed that, consistent with previous reports (14, 31, 53), the \( E_{50(GTP)} \) was reduced from its normal value of 60 μM down to 25 μM in the presence of 100 μM BAY 41-2272 and 50 nM NO (Fig. 10A).

The allosteric enhancer is designated with the letter Y (after YC-1, the prototype compound of this class), \( R \), the receptor protein. Equilibrium constants or microscopic rate constants for the binding and isomerization steps are indicated; values not given are the same as in Fig. 28.

FIGURE 8. Model for allosteric enhancers. The allosteric enhancer is designated with the letter Y (after YC-1, the prototype compound of this class). \( R \), the receptor protein. Equilibrium constants or microscopic rate constants for the binding and isomerization steps are indicated; values not given are the same as in Fig. 28.

The allosteric model accurately replicates the BAY 41-2272 concentration-response curve in the presence of 50 nM NO and suggests that the ambient NO concentration (no added NO) during this experiment was near 100 pm (Fig. 9A). The model suggested further testable predictions. First, evidence was presented earlier that, in the absence of BAY 41-2272, reducing the GTP concentration and adding a physiological ATP concentration lowered the potency of NO; a corollary is that the rate of deactivation must be increased, but this is too fast to measure using manual methods. The half-time for deactivation is predicted to be 2.4 s in the presence of 1 mM GTP, 1.5 s with 100 μM GTP, and 0.6 s with 100 μM GTP and 1 mM ATP. In the presence of BAY 41-2272, however, deactivation becomes slowed and easily measured, and, upon combining the two models (Figs. 6 and 8; see below), it is predicted that the changes in deactivation rate upon altering the nucleotide concentrations should be proportionately almost the same as in the absence of BAY 41-2272. In good agreement, the deactivation rate in the presence of BAY 41-2272 was speeded up when GTP was reduced from 1 mM to 100 μM and further speeded by 1 mM ATP (Fig. 9B), the experimentally determined half-times being close to the predicted values.

Another prediction is that an allosteric enhancer should increase the potency of NO. Such an effect has been indicated before (12, 14, 45, 52), but the NO concentrations...
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in these experiments were unknown. One difficulty in obtaining quantitative data even using clamped NO concentrations (as here) is that, at low NO concentrations, it would take such a long time to reach steady-state in the presence of BAY 41-2272 (e.g. about 15 min at 10 μM NO) that measurement is impracticable. Therefore, we conducted the experiment under non-steady-state conditions (2-min exposure) and compared the result with the predicted amount of cGMP formed over the same interval at different NO concentrations. The experiment gave an EC50(NO) value of 54 nM in the presence of 100 μM BAY 41-2272 compared with 1.1 nM in its absence (both at 1 mM GTP), in good agreement with predictions (Fig. 10B). This being so, it is permissible to extrapolate the steady-state EC50(NO) in a saturating concentration of BAY 41-2272 and 1 mM GTP to be 3.8 nM.

Finally, mutational and binding studies together with homology modeling had suggested that a second nucleotide binding site, putatively that of ATP, is also the binding site of the prototype allosteric enhancer YC-1 (14, 43, 54, 55). If so, allosteric enhancers should reduce inhibition by ATP. Investigation using BAY 41-2272 (100 μM) showed that the inhibition normally produced by ATP in concentrations up to 3 mM was lost (Fig. 11A) and that BAY 41-2272 concentration-dependently reversed the inhibition by 3 mM ATP (Fig. 11B). This interaction between BAY 41-2272 and ATP is implicit if the two parts of the mechanism (Figs. 6 and 8) are joined together, so that the allosteric enhancer shifts the equilibrium away from the ATP-bound states. The resulting model predicts the experimental data (Fig. 11).

Activation by CO—On its own, CO is a weak, low efficacy agonist for NO receptors but becomes more potent and with a greatly increased efficacy (similar to that of NO) in the presence of an allosteric enhancer (12). Should the model encapsulate the functioning of the receptor, it should be able to accommodate activation by CO. The precise mechanism of action of CO remains unclear (56), but it appears to induce a low degree of activity by binding to the heme but without the coordinating histidine bond breaking. From studies of a bacterial NO sensor protein, the structural shifts imposed purely by the binding of CO may be enough to increase the likelihood of a conformational change by a sufficient amount to generate a low level of GC activity (57). This was modeled by reducing the forward rates of the efficacy parameters (Ea and EAG in Fig. 2B) by factors of 300, an adjustment that enabled CO to evoke 1% of the maximal NO-stimulated activity. CO binding was modeled using spectroscopic determinations of the association and dissociation rates, which, for a low efficacy agonist like CO, will largely reflect the kinetics of the binding site itself (Fig. 12A and Table 2). The result satisfactorily simulates CO concentration-response curves for GC activity with and without an allosteric enhancer (12), as well as other relevant data (Fig. 12B and Table 2).

DISCUSSION

We have presented a formal mechanism for NO receptor activation and its modulation by GTP, ATP, and YC-1-type allosteric agents and an extension of it that accommodates the agonist properties of CO. The results consolidate a conceptual move away from the protein being considered parenthetically an enzyme that is parenthetically activated by NO to one in which NO binding and catalysis are inextricably linked, just as with agonist binding and signal transduction in any other receptor macromolecule (58). The strong points of the model are that it is minimally biased because it uses few a priori assumptions, yet, at the same time, the permissible values for the equilibrium binding and isomerization constants are greatly constrained by thermodynamics. The advantageous outcome is that the values...
governing the model are almost entirely controlled by experimental data. Reciprocally, the model reproduces quantitatively all of the important functional characteristics of NO (and CO)-evoked GC activity, which, in turn, allows them to be explained.

Usefully, the mechanism can be reduced to the phenomenological scheme depicted in Fig. 1 with reasonable accuracy (see supplemental Fig. S6). This simplification will be helpful in incorporating the NO receptor kinetics into more complex situations, such as simulations of NO diffusion and signaling in cells (20, 59). Mechanistically, however, the simplified version is of limited value. For example, one overt prediction is that by allosterically driving the reaction in which it is consumed, GTP should display cooperative kinetics (a Hill coefficient of at least 1.5), which is not borne out by any data. Rather, as is explicit in the full mechanism, the apparent driving of the reaction by GTP is explained solely by its being a substrate. In a similar way, structural studies of adenylyl cyclase indicate that substrate binding helps to drive the conformational change from the open to closed configuration necessary for catalysis (37).

It has long been known that stimulation with NO causes a decrease in the apparent $K_m$ for GTP, roughly from 150 to 50 $\mu$m (60), but the reasons were unknown. In fact, a change in the apparent $K_m$ for GTP on stimulation is implicit in the proposed mechanism and is explained by there being a continuum of apparent $K_m$ values, ranging from the lowest affinity binding site ($K_G = 188 \mu$m) to the highest ($K_{GA^*} = 25 \mu$m). Structurally, the former would correspond to the open configuration of the catalytic region and the latter to the closed configuration, where additional residues for substrate binding become available (37). Functionally, low NO concentrations work more through the lower affinity site, whereas high NO concentrations push the equilibrium over toward the high affinity site (supplemental Fig. S3B). Only when the efficacy of the receptor is increased (by allosteric enhancement in our experiments) in the presence of a maximal NO concentration does the apparent $K_m$ approximate the affinity of the active catalytic site ($K_{GA^*}$). Other properties of the catalytic site of GC have been less extensively investigated than in the case of adenylyl cyclase, but, based on the kinetic properties and inhibitory effects of the products (31), the mechanism appears very comparable. The model satisfactorily accounts for the features of product inhibition described for the NO receptor-enzyme (see "Results").

The NO binding affinities required to account for the data were 3 and 4 $\mu$m for the GTP-bound and GTP-free receptor, respectively. Direct measurement of these values would be very difficult, but they are in good conformity with results from other approaches, notably the very rapid association rate of NO (9, 10) in combination with an extrapolated half-time for NO dissociation of 5 s at 37 °C in the presence of Mg$^{2+}$ and GTP (8) and the similar measured half-time of 1.6–4 s for deactivation of GC activity at 37 °C (16, 17). The overall dissociation or deactivation rates are composite quantities, but they are expected to be very sensitive to the rate of loss of NO from its binding site. For example, the off-rate for NO in the model (~1 s$^{-1}$) gives an overall dissociation/deactivation half-time of 1.9–2.4 s at 0.25–1 mM GTP (agreeing with experimental findings), and any deviation in the off-rate would produce a proportional change in dissociation/deactivation rate. Hence, there is little scope for the NO binding affinities to differ much from those deduced from the concentration-response data reported here. The NO binding affinity had previously been quoted as 4.2 $\mu$m (61), but this was based on the very slow rate of dissociation observed after exposure to NO in the absence of GTP (8), when the receptor appears to become locked in an inactive NO-bound state (62).

That physiological concentrations of ATP inhibit "soluble" GC activity in homogenates has been known for many years (40), and, more recently, physiological roles for this interaction have been proposed. One speculation based largely on spectral studies has been that ATP, in competition with GTP, converts the receptor into one that has a higher affinity for NO, leading to very slow ligand dissociation and a consequent tonic GC activity (22). However, no functional studies were carried out to test this notion, and subsequent experiments on intact platelets and cerebellar cells failed to find any tonic GC activity after exposure to NO or any high affinity component to its action (21). Reinforcing these negative results from cells, the present experiments showed that, rather than increasing the potency of NO, ATP reduced it, and, instead of slowing deactivation, ATP speeded it up (measured in the presence of BAY 41-2272).
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Another proposal has been that, in cells, NO-activated GC serves as an ATP sensor, matching cGMP output to the prevailing ATP concentration (23). Examination of this hypothesis using the receptor model suggests that, although the level of ATP will certainly affect GC activity, it is not a sensitive ATP detector in that halving or doubling the ATP concentration would only alter the rate of cGMP output by 30–60% (supplemental Fig. S7A).

An alternative role for ATP as an accelerator of NO signal transduction emerges from the present study. Without ATP, the kinetics will be relatively slow. For example, with 0.5 mM NO and 100 μM GTP, activation and deactivation are expected to take about 10 s to be complete. With 1 mM ATP, both rates become 2-fold faster (supplemental Fig. S7B). This kinetic advantage is at the expense of a reduction in maximal GC activity but, according to data from platelets (15) and smooth muscle (63), cells normally have a large receptor reserve, making the trade-off reasonably cost-effective. Moreover, it has been puzzling that NO is about 10-fold less potent at stimulating GC activity in cells (EC50 = 10 nM) than under cell-free assay conditions (15, 21) and that the rate of deactivation in cells upon removal of NO (half-time ~ 0.2 s) is correspondingly 10 times faster (11). Having ATP and GTP at the more physiological concentrations of 1 mM (38, 39) and 100 μM (64–67), respectively, brings the functioning of the purified protein nearer to its cellular equivalent; the EC50 is now 3.4 nM, and the predicted deactivation half-time is 0.6 s. Differences in the ATP and GTP concentrations in cells and those present in the usual cell-free assays can also explain the apparent 4-fold increase in maximal NO-evoked GC activity observed on cell lysis (68).

Finally, we have presented a mechanism for allosteric enhancement of NO receptor function that accounts for all of the principal experimental observations in this and previous studies. In the past, compounds like YC-1 and BAY 41-2272 have been called “NO-independent” activators (18, 69), presumably because they were found to have some activity (albeit small) in the absence of added NO or in the presence of NO scavengers hemoglobin or CPTIO. We find that the potency of NO becomes extremely high in saturating concentrations of BAY 41-2272 (extrapolated EC50 = 4 pM at steady state) so that environmental NO is enough to stimulate activity powerfully and also that CPTIO concentrations higher than the 65 μM used previously (18) are needed to eliminate this NO source. Examination of data from this and other laboratories (18, 51, 52) on the effect of BAY 41-2272 in the absence of added NO suggest an environmentally derived NO concentration of 10–100 pM (cf. Fig. 9A). Considering the amount of NO in the air and its variability, this range falls well within expectations (supplemental Fig. S2).

Various lines of evidence indicate that the allosteric site corresponds to a pocket adjacent to and resembling the catalytic core but lacking key amino acids needed for catalysis (14, 54). The same “pseudosymmetric” site has been purported to be the second nucleotide binding site where ATP acts (43, 55), and our finding that BAY 41-2272 prevents inhibition by ATP (up to 3 mM) is consistent with this postulate. An interaction with ATP helps explain why YC-1 gives a greater enhancement of NO-evoked GC activity in cells than in assays lacking ATP (70–72).

In apparent discord with the proposed mechanism, mutation of amino acids in the vicinity of the pseudosymmetric site led to the conclusion that the allosteric enhancers have two actions, one to increase the maximal response, putatively by acting on the catalytic site, and the other to enhance the NO potency, putatively by affecting NO binding (14). Agonist potency and efficacy are interdependent quantities, however, making interpretation of the effects of mutations problematic (29). In that one mutation (αC594Y) reduced the efficacy and the other (βM537N) increased it (14), the resulting effects on the activity of allosteric enhancers may have more to do with the role that the residues play in the conformational change rather than in binding the compounds.

To conclude, the model presented here replicates all of the main features of NO receptor function as determined using the purified protein and furthers understanding of how it works in cells. It is clearly incomplete, not least because there are a number of other physiological factors that influence GC activity (e.g. free Ca2+ and Mg2+) (73) that have not yet been considered. In addition, the model is drawn up largely from measurements of steady-state NO-evoked activity, and in some situations (e.g. at synapses), this may not be physiological. Except under conditions of allosteric enhancement, however, the key determinants of the overall kinetics should be NO binding and unbinding, and the values in the model for these parameters are likely to be reliable for the purified receptor because they are tightly constrained by experimental data. The 3-fold lower potency of NO and correspondingly faster deactivation measured in cells remain to be accounted for, but a reasonable explanation for both would be that the NO dissociation rate in cells is about 3-fold higher (see supplemental Fig. S6). The core mechanism presented here provides a framework for further understanding the physiological regulation of NO receptors and is likely to be helpful in identifying new avenues for pharmacological intervention.

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