Nitric oxide (NO) signal transduction may involve at least two targets: the guanylyl cyclase-coupled NO receptor (NOGCGR), which catalyzes cGMP formation, and cytochrome c oxidase, which is responsible for mitochondrial O2 consumption and which is inhibited by NO in competition with O2. Current evidence indicates that the two targets may be similarly sensitive to NO, but quantitative comparison has been difficult because of an inability to administer NO in known, constant concentrations. We addressed this deficiency and found that purified NOGCGR was about 100-fold more sensitive to NO than reported previously, 50% of maximal activity requiring only 4 nM NO. Conversely, at physiological O2 concentrations (20–30 μM), mitochondrial respiration was 2–10-fold less sensitive to NO than estimated beforehand. The two concentration-response curves showed minimal overlap. Accordingly, an NO concentration maximally active on the NOGCGR (20 nM) inhibited respiration only when the O2 concentration was pathologically low (50% inhibition at 5 μM O2). Studies on brain slices under conditions of maximal stimulation of endogenous NO synthesis suggested that the local NO concentration did not rise above 4 nM. It is concluded that under physiological conditions, at least in brain, NO is constrained to target the NOGCGR without inhibiting mitochondrial respiration.

Nitric oxide (NO) is a diffusible biological messenger that subserves cell-to-cell signaling functions in most tissues. NO can also be cytotoxic and has been incriminated in many different pathologies, including atherosclerosis, septic shock, cancer, and neurodegenerative disorders (1). Although much has been learned about the mechanism of NO synthesis (2), the transduction pathways engaged by physiological NO signals to modify cell and tissue function remain to be clearly defined.

The established target is the guanylyl cyclase-coupled receptor, or NOGCGR, which exists in at least two different heterodimeric isoforms (α1β1 and α2β1). This is a metabotropic type of receptor equipped with a heme prosthetic group to which NO binds, triggering the formation of cGMP from GTP in the cyclase domain of the protein. Through this route, NO elicits many effects such as smooth muscle relaxation, inhibition of platelet aggregation, and synaptic plasticity (3, 4). Knowledge of the NO concentrations that engage the NOGCGR is important for understanding the receptor kinetics, for informing on the physiological NO concentrations likely to exist in tissues, and for developing realistic models of NO signaling. Currently, however, the information on this issue is incoherent. Studies on the purified α1β1 receptor protein have suggested that the NO concentration giving half-maximal activation (the EC50) is 250 nM (5). More recently, an EC50 of 1.6 μM has been obtained for the enzyme in an extract of rat aorta (6). The validity of this range appears to be supported by several studies that have used the NONOate, diethylenetriamine/N0 adduct (DEA/NO), which degrades to release NO with a half-life of 2.1 min (at 37 °C). Typically the EC50 of DEA/NO in standard assays of NOGCGR activity is about 300 nM (7, 8), and this has been assumed to approximate the potency of NO (8, 9). In contrast to these estimates made on the purified protein or in cell-free extracts, the estimated EC50 value for NOGCGR activation by NO in intact cells from the brain ranges from 20 to 45 nM (10) down to 2 nM (11). Despite the variability on both sides, this comparison might suggest that, along with other functional differences, the NOGCGR in intact cells possesses heightened sensitivity to NO (12).

Another putative target for NO is cytochrome c oxidase, which is the terminal component of the mitochondrial respiratory chain responsible for almost all cellular O2 consumption. By competing with O2 for binding to cytochrome c oxidase, NO is considered to regulate the rate of respiration and the tissue distribution of O2 (13–16). On the other hand, the ensuing inhibition of respiration, and thus, of ATP synthesis, could have pathological repercussions, particularly in tissues such as brain that rely almost entirely on oxidative phosphorylation to meet their energy requirements (17). An important consideration here is the NO concentration range that is active on cytochrome c oxidase relative to that which engages the NOGCGR. Under physiological conditions (30 μM O2), the NO concentration required to achieve 50% inhibition of respiration (the IC50) has been estimated to be 60 nM in brain synaptosomes (18) and 11 nM in isolated mitochondria (19). Hence, depending on which pairs of values are taken, concomitant activation of the NOGCGR and inhibition of mitochondrial respiration.
ration would appear inevitable, or at least plausible.

Aside from the problems of variabilities in the published estimates of the apparent potency of NO toward both these targets, another pertinent issue is that, to determine the true potency of any agonist on its receptor, it is a prerequisite that the agonist be applied in known concentrations that are stable over the period of measurement. Because of the instability of NO in aerobic solutions, particularly in the hyperoxic solutions used in the laboratory, this has not been achieved for either target, raising further uncertainty about their true sensitivities. By devising a method for delivering known, constant NO concentrations, we address this deficiency and provide a quantitative comparison of the potency of NO for activating purified NOGCR and inhibiting mitochondrial respiration.

EXPERIMENTAL PROCEDURES

Constant NO concentrations were obtained by allowing a dynamic equilibrium to exist between NO release from a donor and NO inactivation by red blood cells. Red blood cells were prepared from whole rat blood as described (20) and kept on ice. This stock was diluted to a final concentration of around 2 million cells/ml in either: (a) buffer (pH 7.4) containing NaCl (130 mM), Tris/HCl (50 mM), MgCl₂ (3 mM), EGTA (0.1 mM), and 0.5% bovine serum albumin for NOGCR experiments or (b) a suspension of cells from the cerebellum (20 million cells/ml), prepared as described previously (10), for determination of the sensitivity of mitochondrial respiration to NO.

The NONOates diethylenetriamine/NO adduct (DETA/NO) and DEA/NO (both from Alexis, Nottingham, UK) were prepared in 10 mM NaOH and diluted 1:100 into the final incubation medium. The NO and O₂ concentrations were recorded with a 1-Hz sampling frequency at 37 °C in a sealed, stirred vessel equipped with an electrochemical NO probe (ISO-NOP, World Precision Instruments, Stevenage, UK) and O₂ electrode (Rank Brothers, Cambridge, UK). The rate of O₂ consumption in the presence of fixed NO concentrations achieved following addition of DETA/NO was measured within the O₂ concentration range 20–30 μM. This rate was expressed as a fraction of the control rate measured beforehand within the range 30–40 μM O₂. All measurements were adjusted for slight basilar drift by deducting the gradient found on adding NaCN (1 mM) as described previously (18).

Purified NOGCR (soluble guanylyl cyclase; Alexis) was diluted in a buffer (pH 7.4) containing 10 mM Tris/HCl, 1 mM dithiothreitol, and 0.5% bovine serum albumin to give a stock concentration of 5 μg/ml, which was stored on ice. For experiments with clamped NO concentrations, NOGCR was added to red blood cells that had been incubated at 37 °C. At time 0, substrate (1 mM GTP) at 45 s, and NOGCR (final concentration 0.05 μg/ml) after 1 min. Addition of GTP or the receptor protein did not disturb the steady-state NO concentration, as measured by the electrochemical probe. Aliquots of the reaction mix were removed after various times and inactivated in boiling buffer (50 mM Tris, 4 mM EDTA, pH 7.4). For experiments with DEA/NO, NOGCR was added to a buffer containing: Tris/HCl (50 mM), MgCl₂ (3 mM), EGTA (0.1 mM), GTP (1 mM), dithiothreitol (3 mM), and 0.5% bovine serum albumin at 5 s before addition of DEA/NO. Aliquots were removed and inactivated in boiling buffer. The levels of cGMP in both cases were measured by radioimmunounassay.

For determining the activity of the NOGCR in brain tissue, 400 μM thick slices of cerebellum from 5-day-old rats (prepared as in Ref. 21) were preincubated for ~2 h in an oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid containing: NaCl (124 mM), KCl (3 mM), NaH₂PO₄ (1.25 mM), MgSO₄ (1 mM), NaHCO₃ (26 mM), CaCl₂ (2 mM), and d-glucose (10 mM) at 37 °C. The slices were exposed to DEA/NO in various concentrations or NMDA (in the presence of 100 μM L-arginine, added 15 min beforehand), and 2 min later, slices were inactivated in boiling buffer (50 mM Tris, 4 mM EDTA, pH 7.4) and then sonicated. The protein content of the homogenate was measured by the bichinchoninic acid method with bovine serum albumin as standard and, following centrifugation, the cGMP content of the supernatant was determined by radioimmunounassay.

RESULTS

Delivering Clamped NO Concentrations—An apparatus for maintaining constant (clamped) NO concentrations has been designed (22), but it is expensive to construct and unsuited to most biological applications. We have found a cheap and simple solution to the problem based on the principle that a fixed NO concentration will be formed when a constant source of NO comes into equilibrium with a "sink" that consumes it. For a source, we used the diethylenetriamine/NO adduct DETA/NO, which decomposes slowly with first order kinetics (a half-life of 20 h at 37 °C), thus releasing NO at an effectively constant rate for many hours. For a sink, we have exploited the ability of oxyhemoglobin (oxyHb) when located within red blood cells to consume NO at an appropriate first order rate, forming methemoglobin and nitrate (20). When DETA/NO (50–500 μM) was mixed with a suspension of red blood cells (2 million/ml), a fixed concentration of NO was attained within 1 min (Fig. 1a). The amplitude of the steady-state concentration varied faithfully with the rate of NO release so that a linear relationship was observed between the DETA/NO concentration and the steady-state NO concentration (Fig. 1b). At the upper extreme (750 μM DETA/NO), the NO sink became exhausted after around 2 min, presumably due to oxyHb oxidation. Thus, the method is capa-

![FIG. 1. Clamping the NO concentration.](image-url)
was well described by the Hill equation with an EC50 of 3.9 nM. NO concentrations. Data are overlaid with curves calculated from the EC50 values of 250 nM (5) or 1.6
purified NOGCR.
d the product remained stable in the mixture (Fig. 1 tration (Fig. 1 cGMP resulting from receptor activation accumulated linearly
not affect the NO concentration (not shown). Accordingly, the maximal response observed in each experiment.
ble of producing clamped NO concentrations covering the range 0–75 nM and lasting at least several minutes. This simple reaction mixture was used to test NOGCR sensitivity, but in principle, introduction of an appropriate concentration of red blood cells in suspension into any biological system would enable NO clamping to be achieved.
Sensitivity of the NOGCR to NO—Addition of small volumes (10 μl) of purified NOGCR to this pre-equilibrated mixture (1 ml) in the presence of substrate (GTP) and co-factor (Mg2+) did not affect the NO concentration (not shown). Accordingly, the cGMP resulting from receptor activation accumulated linearly with time and with no visible delay, regardless of NO concentration (Fig. 1c). Furthermore, addition of cGMP showed that the product remained stable in the mixture (Fig. 1d). The full concentration-response curve (Fig. 2a) had a threshold of ~0.5 nM, and maximum activity was observed at ~20 nM. The curve was well described by the Hill equation with an EC50 of 3.9 nM and a Hill constant of 2.1.
The high sensitivity of the purified NOGCR to NO found here contrasts with previous measurements variously reporting EC50 values of 250 nM (5) or 1.6 μM (6), possible reasons for which are discussed later (see “Discussion”).

Estimating the Maximal Endogenously Produced NO Concentration—The O2 consumption of cells from rat brain (cerebellum) was measured in the presence of fixed NO concentrations and at an O2 concentration of between 20 and 30 μM, corresponding to the range typically found in the brain in vivo (17). As expected, concentration-dependent inhibition was observed, but the IC50 value was about 120 nM (Fig. 4a,b), which is 2–10-fold higher than found in earlier work on brain synaptosomes and isolated mitochondria (18, 19). As with the NOGCR, the Hill constant was 2, which is in agreement with findings using isolated mitochondria (19).
The question was then addressed from a different perspective: how low does the O2 concentration need to be before an NO concentration that is maximally effective on the NOGCR (20 nM) exerts significant respiratory inhibition? For 50% inhibition by 20 nM NO, the O2 concentration needed to fall to 5 μM (Fig. 4c).

Sensitivity of Mitochondrial Respiration to NO—The O2 consumption of cells from rat brain (cerebellum) was measured in the presence of fixed NO concentrations and at an O2 concentration of between 20 and 30 μM, corresponding to the range typically found in the brain in vivo (17). As expected, concentration-dependent inhibition was observed, but the IC50 value was about 120 nM (Fig. 4a,b), which is 2–10-fold higher than found in earlier work on brain synaptosomes and isolated mitochondria (18, 19). As with the NOGCR, the Hill constant was 2, which is in agreement with findings using isolated mitochondria (19).

The question was then addressed from a different perspective: how low does the O2 concentration need to be before an NO concentration that is maximally effective on the NOGCR (20 nM) exerts significant respiratory inhibition? For 50% inhibition by 20 nM NO, the O2 concentration needed to fall to 5 μM (Fig. 4c).
exposed to increasing concentrations of exogenous NO, using DEA/NO as the donor. Because of the powerful NO inactivation pathway that is expressed in brain and other tissues, high concentrations of NO donors need to be applied to intact brain slices to supply the cells with active concentrations of free NO (11). At the peak of the response to DEA/NO (100/9262 M; curve not shown), cGMP was 619/11006 33 pmol/mg of protein (n = 5), almost twice the level found with NMDA. The lower response found with NMDA was not because the agonist reduced the responsiveness of the NOGCR (e.g., through raising cytosolic Ca2+; Ref. 26) because simultaneous exposure to NMDA failed to inhibit the response to DEA/NO (717/11006 28 pmol/mg of protein; n = 5). Because pharmacological activation of NMDA receptors in this way will lead to the switch-on of multiple NO sources throughout the slice, it follows that sources and sinks will rapidly come to equilibrium generating a steady-state “continuum” of NO throughout the tissue (27). Visible evidence for the validity of this assumption is provided by immunocytochemical data on cGMP accumulation in NMDA-stimulated cerebellar slices (28). Inspection of Fig. 2a suggests, therefore, that maximal endogenous NO synthesis raises the local NO concentration experienced by NOGCR in situ to about 4 nM.

DISCUSSION

By using clamped NO concentrations, we find that NO is about 100-fold more potent as an agonist for the purified NOGCR and 2–10-fold less potent as an inhibitor of mitochondrial respiration than reported previously, the net effect being that there is minimal overlap in the two concentration-response curves at physiological O2 concentrations (Fig. 4d). This, together with evidence that the NOGCR is not saturated during maximal endogenous NO synthase activity and that even a saturating NO concentration for the NOGCR only affects respiration when the O2 concentration is very low, signifies that, in the brain at least, the NO signaling pathway has evolved to target the NOGCR without simultaneously influencing mitochondrial function.

The explanation for the much higher previous estimates of

![Fig. 3. Time courses of NOGCR activity and NO concentration following addition of DEA/NO.](image-url)

- **a**. NO concentration profile (mean of three traces) and cGMP accumulation (mean ± S.E., n = 3) evoked by 300 nM DEA/NO, a half-maximally effective concentration when a 10-min incubation period is used. The two sets of data were obtained in parallel. The periods where the NO concentration lies within the sensitivity range of the NOGCR (as determined using clamped NO concentrations; Fig. 2a) are shaded. Synthesis of cGMP proceeds at a maximal rate (dashed line; mean rate = 5.5 ± 0.7 μmol/mg of protein/min, n = 9, from experiments with clamped NO concentrations) until the NO level falls below ~20 nM. The solid line gives the activity profile predicted assuming an EC50 of 3.9 nM and maximum activity of 5.5 μmol/mg of protein/min. **b**, time course for receptor deactivation after abrupt removal of free NO with oxyHb. NO was clamped by a DETA/NO-red blood cell mixture at a concentration supramaximal for the NOGCR (representative traces in upper panel) and the receptor activity recorded in parallel (lower panel). Addition of oxyHb (○) caused an immediate cessation of cGMP synthesis; the slower apparent decline in the corresponding trace of the NO concentration (upper panel) reflects the probe response time. In the absence of oxyHb (●), NO and NOGCR activity were maintained. Data in the lower panels are mean ± S.E. (n = 4).
the potency of NO on the NOGC-R in its purified form or in extracts (0.25 and 1.6 μM; Refs. 5 and 6) is not entirely clear, but the NO concentrations were not measured over time, and in one case (5), losses occurring through reaction with components of the reaction mixture, such as dithiothreitol and residual O₂, presumably exceeded expectations. In the other case (6), the value of 1.6 μM was derived by summing the NO released by various donors over a 10-min period, disregarding NO loss or

FIG. 4. Sensitivity of mitochondrial respiration to NO. a, simultaneous measurement of O₂ consumption (upper traces; smoothed by 10-point adjacent averaging) and NO concentration (lower traces; unsmoothed) in response to various DETA/NO concentrations added to a mixture of cerebellar cells (20 million/ml) and red blood cells (2 million/ml) to give clamped NO concentrations (values given beside each trace). Note that the range of NO concentrations that could be maintained in this way was larger than that shown in Fig. 1a because additional NO inactivation was provided by the cerebellar cells (11). b, concentration-response curve for inhibition of respiration by NO. The curve was obtained by binning the NO concentrations into regularly spaced, non-overlapping groups (each containing three to eight individual measurements). The solid line fits the data to the Hill equation with a coefficient of 2. Error bars are S.E. c, the O₂ consumption of cerebellar cells (20 million/ml) was measured in the presence of red blood cells (2 million/ml) with (dark traces) or without (light traces) addition of 150 μM DETA/NO to give a fixed NO concentration of 20 nM (lower traces). Addition of oxyHb (10 μM) at the end removed free NO and caused a small rise in O₂ concentration (due to O₂ in the oxyHb solution) and a resumption of respiration until all the O₂ had gone. Both sets of traces are means of three runs with the S.E. at every 30 s time point added as vertical bars to the upper set. d, comparison of the concentration-response relationships of NOGC-R (solid line) and cytochrome c oxidase (broken line). Lines are fits to the data in Figs. 2a and Fig. 4b.
degradation altogether. As shown here using DEA/NO, the 
EC_{50} value derived in this way is misleading because it measures the donor concentration required to maintain NO in active concentrations for half the incubation period, not the potency of NO on its receptor. It follows that changes (or otherwise) in the EC_{50} value for the donor cannot alone be taken as evidence for changes (or otherwise) in the sensitivity of the NOGC\(\text{R}\). The recent conclusion, made on this basis, that association with membranes “sensitizes” the receptor to NO (29) may therefore be spurious as a lower rate of NO consumption (16, 30) by membrane fractions as compared with cytosol could account for the differing apparent EC_{50} values for DEA/NO (0.24 versus 0.48 \(\mu M\), respectively).

On the other hand, the EC_{50} value measured here for the purified NOGC\(\text{R}\) (4 \(\mu M\)) is in good agreement with the value of 2 \(\mu M\) estimated for intact brain cells (11), especially considering the large extrapolation involved in arriving at this estimate. Higher EC_{50} values (20–45 \(\mu M\)) have also been reported for the same cells (10), but these relied on assumptions about the efficiency of NO release from a caged precursor, which must be regarded as questionable. Consequently, there are now no good grounds for proposing the existence of cellular factors that enhance the potency of NO on its receptor (12). Furthermore, in our hands, the rapid rate of deactivation of purified NOGC\(\text{R}\) (\(\approx 0.5 s\), Fig. 3b) is consistent with the subsecond deactivation rate measured in intact cells (10). Although the reasons for the much slower rates (from 5 \(s\) to 3 \(min\)) found for the purified NOGC\(\text{R}\) in other laboratories (9, 23, 24) and their variability remain uncertain, this finding dissipates the need to invoke the existence of factors in cells that enhance the rate of dissociation of NO from its receptor (9, 10, 12).

In addition to providing a measure of the potency of NO for the NOGC\(\text{R}\), the shape of the concentration-response curve is also instructive. The current model of a single binding event to the heme prosthetic group (3) predicts a Hill slope of 1 (Fig. 2b, dashed line), whereas we found that the curve was steeper, with a Hill slope of 2. The most straightforward interpretation of a slope greater than 1 is that the receptor incorporates multiple agonist binding sites that act in a cooperative fashion. This possibility has been raised previously for the NOGC\(\text{R}\) based on analysis of activation kinetics (31), but we have recently argued that these data are equivocal and can be fitted by a simpler model (32). The shape of the equilibrium concentration-response curve of the NOGC\(\text{R}\) provides the first unambiguous evidence that a single NO binding event cannot underlie receptor activation. It follows that the simplest model must now incorporate two NO binding events, meaning that the NOGC\(\text{R}\) can exist in at least four states (unbound, a single NO-bound state, a double NO-bound state, and an active state) and that progressive activation of a population of receptors would proceed in three kinetic phases. Testing the predictions of this new model against existing equilibrium and kinetic data is clearly desirable.

In addition to NOGC\(\text{R}\) activation, there has been growing interest in the possibility that NO could function as a regulator of mitochondrial respiration, based in part on the assumption that at physiological \(O_2\) concentrations, cytochrome \(c\) oxidase would be inhibited by NO concentrations similar to those required for NOGC\(\text{R}\) activation (13–15). Our experiments, however, showed that the two concentration-response curves were separated by a factor of 30 and that the IC_{50} for respiratory inhibition by NO was 2–10-fold higher than previous estimates (18, 19). The discrepancy probably relates to the former use of bolus-like additions of NO because when the brain cells used here were challenged with boluses of NO (at \(\approx 100 \mu M\) \(O_2\)), the IC_{50} measured during the subsequent decay of NO (120 nm; Ref. 11) matched that found for isolated mitochondria (also at \(\approx 100 \mu M\) \(O_2\)) using a similar approach (19). Based on this result, it would have been predicted that the IC_{50} in brain cells would have been about 11 \(nM\) at 30 \(\mu M\) \(O_2\) (19), whereas with clamped NO concentrations, it was 10-fold higher (120 nm). Presumably, inadvertent effects of the bolus itself and/or the use of ever-changing concentrations during the decay phase account for the different values. In any case, boluses of NO are unlikely to be found in vivo because the relatively slow switch-on of NO synthesis, which is dependent on \(Ca^{2+}\)-calmodulin interactions or phosphorylation cascades (2), would be quickly balanced by NO inactivation (predicted to impose a half-life on NO of about 100 ms; Refs. 11 and 16), the result being a steady-state NO concentration analogous to that formed by the DETA/NO-red blood cell mixture (Figs. 1a and 4a) but much faster (a 100-ms time scale).

It was only with \(O_2\) at very low concentrations that an NO concentration maximally active on the NOGC\(\text{R}\) (20 nm) could affect respiration. The \(O_2\) concentration needed for 50% inhibition under these conditions (5 \(\mu M\)) corresponds to one that, if sustained for more than a few seconds in brain tissue, is pathological (17). Indeed, initial signs of metabolic stress occur in brain at about 10 \(\mu M\) \(O_2\) (17), at which concentration 20 nm NO failed to affect respiration (Fig. 4c). Moreover, given the high \(K_m\) value of the brain NO synthase for \(O_2\) (350 \(\mu M\); Ref. 33), it is doubtful that 20 nm NO and low micromolar \(O_2\) could coexist unless the pathway that inactivates NO (11) is compromised. However, although also \(O_2\)-dependent (30), this pathway is as active at 30 \(\mu M\) \(O_2\) as it is at 200 \(\mu M\) \(O_2\), indicating a much lower \(K_m\) value than the NO synthase.\(^3\)

From these data, it is unlikely that NO concentrations in the range active on the NOGC\(\text{R}\) could simultaneously inhibit mitochondrial respiration in vivo. This raises the question of whether NO ever rises above this range physiologically to the extent that it could influence \(O_2\) consumption. Our attempt to answer this by maximal stimulation of endogenous NO formation in brain slices suggested that this was not the case in that local NO rose only to an apparent concentration of 4 nm. This is consistent with studies in the brain in vivo where, even under conditions of abnormally elevated neuronal activity, cGMP rises to only a fraction of the maximum levels achievable (34). Furthermore, from a recent spectroscopic analysis of cytochrome \(c\) oxidase in rat brain in vivo, it has been concluded that endogenous NO does not inhibit this enzyme, either during physiological conditions or during reperfusion following transient ischemia (35). This finding accords with our data suggesting that NO rises only to about 1 nm in slices of rat striatum in vitro subjected to simulated ischemia and reperfusion (36). The NO-consuming pathway identified recently in brain (11) appears well suited to constrain NO concentrations to the low nanomolar range that selectively engages the NOGC\(\text{R}\). Whether or not NO is similarly constrained in other tissues remains to be determined.

Finally, the NO concentrations active on the NOGC\(\text{R}\) have long been used as a guide to the physiological NO concentrations existing in tissues, and thus, to the exogenous concentrations that should be applied experimentally. Our results suggest a downward revision by about 2 orders of magnitude in this range. As a consequence, many findings made using NO concentrations previously considered physiological may be of more pathological relevance, although what constitutes a pathological NO concentration in vivo remains to be defined.

\(^3\) C. Griffiths and J. Garthwaite, unpublished observation.
REFERENCES

1. Hobbs, A. J., Higgs, A., and Moncada, S. (1999) Annu. Rev. Pharmacol. Toxicol. 39, 191–220
2. Stuehr, D. J. (1999) Biochim. Biophys. Acta 1411, 217–230
3. Koesling, D., and Frühe, A. (1999) Rev. Pharmacol. Biochem. Pharmacol. 135, 41–65
4. Garthwaite, J., and Boulton, C. L. (1995) Annu. Rev. Physiol. 57, 683–706
5. Stone, J. R., and Marletta, M. A. (1996) Biochemistry 35, 1093–1099
6. Artz, J. D., Toader, V., Zavorin, S. I., Bennett, B. M., and Thatcher, G. R. (2001) Biochemistry 40, 9256–9264
7. Schrammel, A., Behrends, S., Schmidt, K., Koesling, D., and Mayer, B. (1996) Mol. Pharmacol. 50, 1–5
8. Ruszwurm, M., Behrends, S., Harteneck, C., and Koesling, D. (1998) Biochem. J. 335, 125–130
9. Ruszwurm, M., Mergia, E., Mullershausen, F., and Koesling, D. (2002) J. Biol. Chem. 277, 24883–24888
10. Bellamy, T. C., and Garthwaite, J. (2001) J. Biol. Chem. 276, 4287–4292
11. Griffiths, C., and Garthwaite, J. (2001) J. Physiol. (Lond.) 536, 855–862
12. Bellamy, T. C., Wood, J., Goodwin, D. A., and Garthwaite, J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2928–2933
13. Moncada, S., and Erusalimsky, J. D. (2002) Nature Rev. Mol. Cell. Biol. 3, 214–220
14. Cooper, C. E. (2002) Trends Biochem. Sci. 27, 33–39
15. Brown, G. C. (2001) Biochim. Biophys. Acta 1504, 46–57
16. Thomas, D. D., Liu, X., Kuntrow, S. P., and Lancaster, J. R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 355–360
17. Ecenaisa, M., and Silver, I. A. (2001) Respir. Physiol. 128, 263–276
18. Brown, G. C., and Cooper, C. E. (1994) FEBS Lett. 356, 205–208
19. Kivistö, A., Matthias, A., Brumnikov, G., and Nedergaard, J. (1997) FEBS Lett. 417, 75–80
20. Liu, X., Miller, M. J., Joshi, M. S., Sadowska-Krowicka, H., Clark, D. A., and Lancaster, J. R., Jr. (1998) J. Biol. Chem. 273, 18709–18718
21. Southam, E., East, S. J., and Garthwaite, J. (1991) J. Neurochem. 56, 2072–2081
22. Zhelyaskov, V. R., and Godwin, D. W. (1999) Nitric Oxide 3, 419–425
23. Brandish, P. E., Buetchler, W., and Marletta, M. A. (1998) Biochemistry 37, 16898–16907
24. Kharitonov, V. G., Ruszwurm, M., Magde, D., Sharma, V. S., and Koesling, D. (1997) Biochem. Biophys. Res. Commun. 239, 284–286
25. Garthwaite, J., Charles, S. L., and Chess Williams, R. (1988) Nature 336, 385–388
26. Parkinson, S. J., Jovanovic, A., Jovanovic, S., Wagner, F., Terzic, A., and Waldman, S. A. (1999) Biochemistry 38, 6441–6448
27. Wood, J., and Garthwaite, J. (1994) Neuropharmacology 33, 1235–1244
28. de Vente, J., Bl, J. G. J. M., Berkelmans, H. S., Schipper, J., and Steinbusch, H. M. W. (1990) Eur. J. Neurosci. 2, 845–862
29. Zabel, U., Kleinschmit, C., Oh, P., Nedvetsky, P., Smolenski, A., Muller, H., Kronich, P., Kugler, P., Walter, U., Schnitzer, J. R., and Schmidt, H. H. (2002) Nat. Cell Biol. 4, 397–411
30. Griffiths, C., Yamini, B., Hall, C., and Garthwaite, J. (2002) Biochem. J. 362, 459–464
31. Zhao, Y., Brandish, P. E., Ballou, D. P., and Marletta, M. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14753–14758
32. Bellamy, T. C., Wood, J., and Garthwaite, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 607–610
33. Santolini, J., Meade, A. L., and Stuehr, D. J. (2001) J. Biol. Chem. 276, 48887–48898
34. Wood, P. L. (1991) Pharmacol. Rev. 43, 1–25
35. De Visscher, G., Springett, R., Delpy, D. T., Van Reempts, J., Borgers, M., and van Rossem, K. (2002) J. Cereb. Blood Flow Metab. 22, 515–519
36. Griffiths, C., Garthwaite, G., Goodwin, D. A., and Garthwaite, J. (2002) Eur. J. Neurosci. 15, 962–968
37. Ruszwurm, M., Wittau, N., and Koesling, D. (2001) J. Biol. Chem. 276, 44447–44452