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Formation of nitric oxide from L-arginine in the central nervous system: A transduction mechanism for stimulation of the soluble guanylate cyclase

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ABSTRACT **A soluble enzyme obtained from rat forebrain catalyzes the NADPH-dependent formation of nitric oxide (NO) and citrulline from L-arginine. The NO formed stimulates the soluble guanylate cyclase and this stimulation is abolished by low concentrations of hemoglobin. The synthesis of NO and citrulline is dependent on the presence of physiological concentrations of free Ca^{2+} and is inhibited by N^G-monomethyl-L-arginine, but not by its enantiomer N^G-monomethyl-D-arginine or by L-canavanine. L-Homoarginine, L-arginyl-L-aspartate, or L-arginine methyl ester can replace L-arginine as substrates for the enzyme. These results indicate that NO is formed from L-arginine in the brain through an enzymic reaction similar to that in vascular endothelial cells, neutrophils, and macrophages, adding support to our hypothesis that the formation of NO from L-arginine is a widespread transduction mechanism for the stimulation of the soluble guanylate cyclase.**

Vascular endothelial cells synthesize nitric oxide (NO) from the terminal guanido nitrogen atom(s) of L-arginine, and this accounts for the biological activities attributed to endothelium-derived relaxing factor (EDRF; refs. 1–3). The actions of NO are the result of stimulation of the soluble guanylate cyclase in vascular smooth muscle, platelets, and probably other cells (for review see ref. 4).

Activated macrophages also synthesize NO_2^- and NO_3^- from the terminal guanido nitrogen atom(s) of L-arginine (5). This reaction, which occurs via the formation of NO (6), is involved in the cytotoxic activities of these cells (7). We have demonstrated recently that rat peritoneal neutrophils form NO from L-arginine (8).

The endothelial cell and macrophage enzyme that forms NO from L-arginine is soluble, is NADPH-dependent, forms citrulline as a coproduct, and is inhibited by N^G-monomethyl-L-arginine (L-MeArg; refs. 6 and 9). Furthermore, in both cells the enzyme requires a divalent cation, which in the case of the macrophage has been suggested to be Mg^{2+} (6).

Some years ago, L-arginine was identified as an endogenous activator of the soluble guanylate cyclase in brain tissue (10). Since this activation resembled that of the nitrovasodilators (11) and NO is known to stimulate soluble guanylate cyclase in the brain (12), we have investigated the existence in the central nervous system of an enzymic system capable of converting L-arginine into NO and citrulline.

While this work was in progress Garthwaite *et al.* (13) reported that *N*-methyl-D-aspartate, an excitatory amino acid known to elevate cyclic GMP levels in the brain, induces the release of an EDRF-like material from rat cerebellar cells.

MATERIALS AND METHODS

Materials. L-[2,3,4,5-^{3}H]Arginine (57 Ci/mmol; 1 Ci = 37 GBq) and cyclic GMP radioimmunoassay kits were obtained from Amersham. NO (British Oxygen) was prepared as a 3% (vol/vol) solution as described (1). L-MeArg and D-MeArg were synthesized as described (14). Chelating resin (sodium form, 50–100 mesh, catalogue no. C7901) was obtained from Sigma. Other chemicals were obtained from Sigma or Aldrich.

Preparation of Crude Synaptosomal Cytosol. Male rats (200–300 g, four for each preparation) were killed by cervical dislocation and the forebrains were rapidly removed and cooled in ice-cold washing buffer (0.32 M sucrose/10 mM Hepes/0.1 mM EDTA, pH 7.4); subsequent procedures were carried out at 0–4°C. The tissue was placed in fresh washing buffer, finely minced, washed once with 50 ml of washing buffer and twice with homogenization buffer (0.32 M sucrose/10 mM Hepes, 1 mM DL-dithiothreitol, pH 7.4) to remove contaminating erythrocytes, and homogenized with 20 strokes of a Dounce homogenizer. The homogenate was diluted to 50 ml with homogenization buffer and centrifuged (1400 × *g*, 10 min); the supernatant obtained was centrifuged (18,000 × *g*, 10 min) to obtain a crude synaptosomal pellet. After aspiration of the supernatant, 8 ml of 1 mM DL-dithiothreitol (dissolved in distilled water) was added to the pellet to cause hypotonic swelling of the synaptosomes, which were then lysed by homogenization as above. The soluble fraction was obtained as the supernatant from centrifugation at 150,000 × *g* for 30 min. This soluble fraction was passed through a 2-ml column of chelating resin to remove endogenous divalent cations and arginine (11), and the synaptosomal cytosol was made up to 10 ml with 1 mM DL-dithiothreitol and stored on ice until used.

Determination of Cyclic GMP, NO, and Citrulline Formation. Incubations for cyclic GMP formation were initiated by addition of 150 μl of synaptosomal cytosol to prewarmed (37°C) buffer, to give (final concentrations) 25 mM Tris/5 mM GTP/5 mM $MgCl_2$/1 mM 3-isobutyl-1-methylxanthine, pH 7.2 at 37°C, in a total volume of 200 μl. Incubations were terminated by addition of 20 μl of 20% $HClO_4$ and cooling on ice. Following centrifugation (12,000 × *g*, 1 min), the cyclic GMP was determined by radioimmunoassay in aliquots of the supernatants diluted 1:100 in 25 mM Tris/4 mM EDTA, pH 7.2 at 0°C.

NO was measured as its breakdown product, NO_2^-, following incubation as described above but terminated by the addition of EGTA to 1 mM and cooling on ice. NO_2^- formed was determined by chemiluminescence, as described (1); in brief, 50-μl aliquots of incubates were injected into a reaction vessel containing 75 ml of 1% NaI in glacial acetic acid, the NO formed was removed under reduced pressure in a stream of nitrogen and mixed with ozone, and the resulting chemiluminescence was measured. The NO released was quantified by reference to NO standards.

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Abbreviations: EDRF, endothelium-derived relaxing factor; MeArg, N^G-monomethylarginine; SNAP, *S*-nitroso-*N*-acetylpenicillamine.

Citrulline formation was determined in incubates that also included L-[^{3}H]arginine (10 μCi/ml, 0.2 μM); incubation was terminated with $HClO_4$ as above. The stimulation of guanylate cyclase at this concentration of L-arginine was below the limit of detection of this method. This low concentration of arginine was necessary in these experiments to maximize the specific activity of the L-[^{3}H]arginine and therefore the sensitivity of the [^{3}H]citrulline determination. Aliquots (100 μl) of supernatants were fractionated by ion-exchange high-pressure liquid chromatography and the radioactivity in fractions corresponding to L-arginine, L-ornithine, and L-citrulline was determined by liquid scintillation counting as described (9).

Incubations at defined free Ca^{2+} concentrations were carried out in the presence of 1 mM EGTA and up to 1.0 mM added $CaCl_2$; the resulting free Ca^{2+} concentrations were calculated as described by Fabiato and Fabiato (15), allowing for the presence of Mg^{2+} and GTP and using the following apparent stability constants (M^{-1}) for the measured final pH (7.16) of such incubations: Ca^{2+}-EGTA, 5.22×10^6; Ca^{2+}-GTP, 5.96×10^3; Mg^{2+}-EGTA, 63.2; Mg^{2+}-GTP, 13.3×10^3.

Results are expressed as mean ± SEM of n separate experiments. Statistical significance was determined by Student's t test, and $P < 0.05$ taken as significant.

RESULTS

Synthesis of NO and Citrulline. Addition of L-arginine (100 μM) and NADPH (1 mM) to incubations containing synaptosomal cytosol induced the synthesis of NO (40 ± 5.8 pmol/min per ml; $n = 4$), determined as its breakdown product NO_2^-. The L-arginine-dependent NO_2^- production was abolished by hemoglobin (1 μM, $n = 3$). The synthesis of NO was accompanied by stimulation of guanylate cyclase with half-maximal stimulation observed at 6 ± 2 μM L-arginine and saturation at ≈50 μM ($n = 3$). This stimulation was similar in magnitude to that induced by NO, sodium nitroprusside, or *S*-nitroso-*N*-acetylpenicillamine (SNAP) and was also abolished by hemoglobin (Table 1).

The guanylate cyclase stimulation by L-arginine (100 μM) was maximal in the presence of exogenous NADPH (1 mM). In its absence the stimulation was only 40 ± 11.4% ($n = 7$) of that in its presence. This variation presumably reflects variability in the endogenous NADPH content of the synaptosomal cytosol preparations.

Incubation of synaptosomal cytosol with 0.2 μM L-[^{3}H]arginine and 1 mM NADPH also resulted in the synthesis of [^{3}H]citrulline (44.9 ± 11.0 fmol/min per ml, $n = 4$). The rate of synthesis of citrulline from L-arginine was NADPH-dependent; the rate in the absence of added NADPH was only 26 ± 7.0% ($n = 4$) of that in its presence. [^{3}H]Ornithine synthesis was not observed ($n = 3$).

Table 1. Stimulation of guanylate cyclase in synaptosomal cytosol by L-arginine, NO, and nitroso compounds

Addition	Concentration, μM	Guanylate cyclase activity, nmol/min per ml
None	—	0.03 ± 0.01
Nitric oxide	33	0.99 ± 0.22*
Sodium nitroprusside	100	0.95 ± 0.19*
SNAP	10	1.15 ± 0.07*
L-Arginine	100	0.69 ± 0.22*
L-Arginine + hemoglobin	100 + 1	0.02 ± 0.01

Guanylate cyclase activity in synaptosomal cytosol in the presence of various additions was determined over a 10-min incubation, as described in *Materials and Methods*. NO was added to incubation mixtures immediately after the addition of synaptosomal extract.
*$P < 0.05$ vs. the activity with no additions ($n = 4$).

The time courses of NO formation, measured by guanylate cyclase stimulation and citrulline formation, were similar, reaching a maximum after 10–20 min ($n = 3$). Guanylate cyclase stimulation in the presence of L-arginine was linearly related to the square root of the rate of citrulline formation (Fig. 1).

Ca^{2+} Dependence of NO and Citrulline Synthesis. When the free Ca^{2+} concentration in the incubations was controlled by Ca^{2+}/EGTA buffers, the activity of the guanylate cyclase measured in the presence of either SNAP (10 μM) or NO (33 μM) was found to be inhibited by free Ca^{2+} concentrations above 80 nM, with a half-maximal effect at 120 nM and complete inhibition at 1.5 μM. Because of this, the stimulation of the guanylate cyclase by L-arginine at different free Ca^{2+} concentrations was expressed as a percentage of the stimulation induced by 10 μM SNAP.

The synthesis of both NO and citrulline from L-arginine was found to be entirely Ca^{2+}-dependent (Fig. 2). Half-maximal rates were observed at ≈160 nM and ≈170 nM, respectively, and no synthesis of either NO or citrulline was detectable below ≈80 nM free Ca^{2+}.

Substrate and Inhibitor Specificity of the Enzyme. NO synthesis, measured by guanylate cyclase stimulation, was also observed when L-arginyl-L-aspartate, L-arginine methyl ester, or L-homoarginine was substituted for L-arginine (Table 2). The concentrations of these three compounds causing half-maximal stimulation were 6 ± 0.4 μM ($n = 3$), 25.4 ± 16.4 μM ($n = 3$) and 174 ± 46.5 μM ($n = 7$), respectively.

L-MeArg inhibited the synthesis of NO when measured as its degradation product NO_2^- or as stimulation of the guanylate cyclase. At a concentration of 200 μM, L-MeArg inhibited the L-arginine (20 μM)-dependent stimulation of the guanylate cyclase by 93 ± 3.5% ($n = 7$) and the formation of NO_2^- by 116 ± 11.4% ($n = 4$). The inhibition by L-MeArg of the

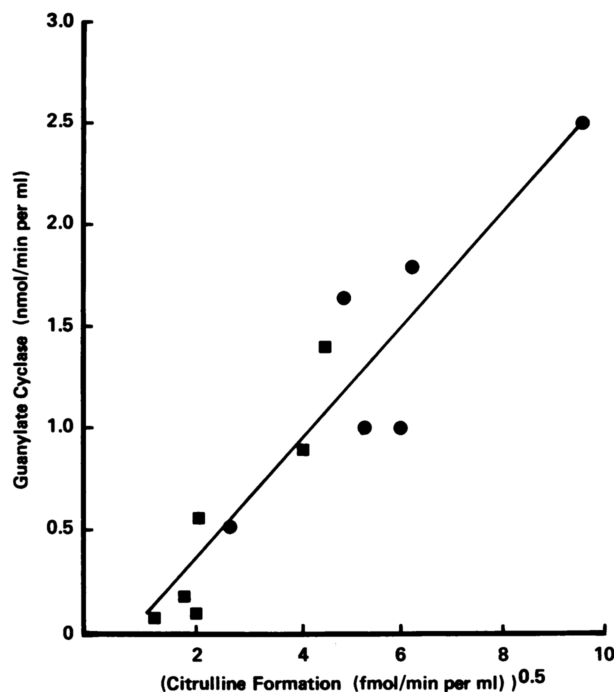

FIG. 1. Relationship between the formation of NO and citrulline from L-arginine. The stimulation of guanylate cyclase in the presence of 100 μM L-arginine and the formation of [^{3}H]citrulline from 0.2 μM L-[^{3}H]arginine were determined in 10-min incubations (see *Materials and Methods*). Data are shown from six separate enzyme preparations incubated in the presence (●) or absence (■) of added NADPH (1 mM). The line shown was obtained by linear regression: $y = 0.289x - 0.221$, $r = 0.920$, $n = 12$.

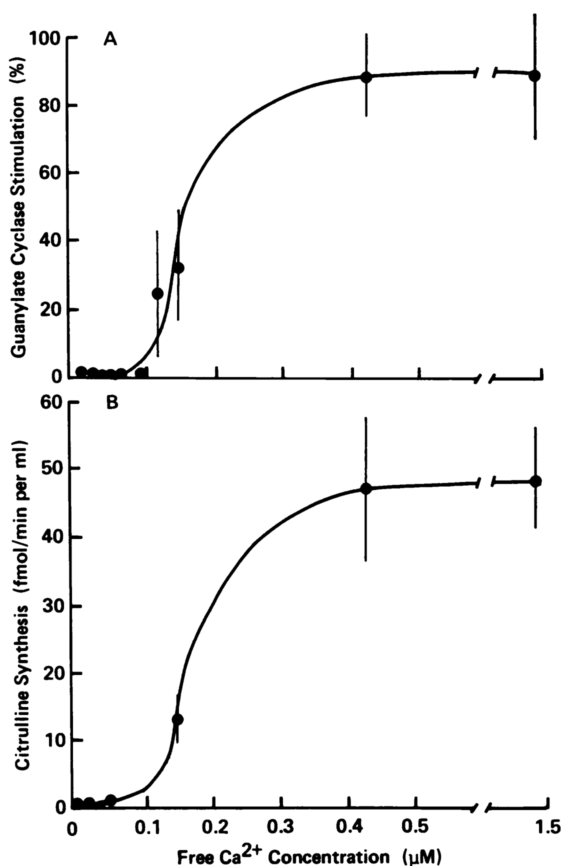

FIG. 2. Ca^{2+} dependence of NO and citrulline synthesis from L-arginine. The rates of synthesis of NO from 100 μM L-arginine at free Ca^{2+} concentrations defined by Ca^{2+}/EGTA buffers as determined by stimulation of guanylate cyclase (*A*) or the rates of synthesis of [^{3}H]citrulline from 0.2 μM L-[^{3}H]arginine (*B*) are shown. Since guanylate cyclase activity decreased as the free Ca^{2+} concentration increased, the L-arginine-induced stimulation of guanylate cyclase is expressed as a percentage of that induced by 10 μM SNAP at each free Ca^{2+} concentration. The results shown are from three to six experiments.

guanylate cyclase stimulation by the different substrates was concentration-dependent with a half-maximal inhibition observed with 6 ± 2.0 μM for L-arginine (20 μM), 21 ± 7.2 μM for L-arginyl-L-aspartate (20 μM), 7 ± 0.7 μM for L-arginine methyl ester (20 μM), and 22 ± 3.5 μM for L-homoarginine (200 μM; n = 3 for each). L-MeArg (20 μM) also inhibited the formation of [^{3}H]citrulline from 0.2 μM L-[^{3}H]arginine by 93 ± 2.5% (n = 4), whereas D-MeArg (20 μM) had no significant effect.

Table 2. Effects of L-arginine analogs and other compounds on guanylate cyclase activity in soluble extracts of synaptosomes

Compound	Activity
Substrates	(% of control)
L-Homoarginine	57.0 ± 7.2 (4)*
L-Arginyl-L-aspartate	106 ± 29.9 (4)*
L-Arginine methyl ester	95 ± 17.3 (4)*
D-Arginine	10 ± 4.3 (3)
L-Citrulline	17 ± 11.2 (4)
Ammonium chloride	12 ± 5.8 (3)
L-α-Amino-γ-guanidinobutyrate	1 ± 0.5 (4)
N^{α}-Benzoyl-L-arginine ethyl ester	12 ± 6.1 (4)
Inhibitors	(% inhibition)
L-MeArg	93 ± 3.5 (7)*
D-MeArg	13 ± 6.5 (6)
L-Canavanine	12 ± 3.2 (3)
Formamidine acetate	10 ± 2.3 (3)

The activity of compounds (200 μM) as substrates for the formation of NO was determined by incubation for 10 min in the presence of NADPH (1 mM). Results are expressed as a percentage of the activity of 100 μM L-arginine. Their activity as inhibitors of NO synthesis from L-arginine (20 μM) was assessed similarly. The results shown are mean ± SEM (n experiments).
*Significant effect ($P < 0.05$) as substrate or inhibitor.

Stimulation of the soluble guanylate cyclase was not affected by D-arginine, L-canavanine, D-MeArg, L-α-amino-γ-guanidinobutyrate, N^{α}-benzoyl-L-arginine ethyl ester, formamidine acetate, or NH_4Cl (all at 200 μM; Table 2).

DISCUSSION

The brain contains a soluble enzyme that forms NO from L-arginine in the presence of NADPH. This synthesis of NO can be determined either by measuring its breakdown product NO_2^- or by measuring its ability to stimulate soluble guanylate cyclase (16). Both NO_2^- production and guanylate cyclase stimulation were abolished by hemoglobin, which binds NO, and by L-MeArg, which inhibits the NO-forming enzyme in endothelial cells (9) and macrophages (6).

In addition to L-arginine, some L-arginine analogs also stimulated the guanylate cyclase with similar maximal effects, confirming a previous report (11). This finding, together with the observation that their action was inhibited by L-MeArg, indicates that all these compounds are substrates for the NO-forming enzyme.

The concentrations of L-arginine, L-arginyl-L-aspartate, and L-arginine methyl ester required to produce half-maximal stimulation of guanylate cyclase were similar and were substantially less than that for L-homoarginine. Furthermore, L-MeArg was similarly effective as an inhibitor of approximately equally effective concentrations of these substrates. The half-maximally effective concentrations are not apparent K_m or K_i values for the NO-forming enzyme, since we were studying the result of the activity of two enzymes, namely, the NO-forming enzyme and the soluble guanylate cyclase. With the sensitivity of the present method it is not possible to measure NO formation directly at low substrate concentrations, and further work will be necessary to clarify the kinetics of NO formation from L-arginine by the NO-forming enzyme.

Citrulline is also formed from L-arginine by the synaptosomal cytosol. The correlation between NO and citrulline formation in terms of their time course, NADPH and Ca^{2+} dependence, and inhibitor sensitivity suggests that they are coproducts of the same reaction. The observed relationship between NO and citrulline formation strongly supports this suggestion. NO decay is second-order at constant O_2 concentration (17). Therefore, for NO and citrulline to be products of the same reaction, there should be a square-root relationship between the rate of citrulline formation and the NO concentration, provided that the NO concentration is at steady state. Our results are consistent with this.

The NO-synthesizing enzyme from the brain is strikingly dependent on the free Ca^{2+} concentration in the physiological range. The enzyme is essentially inactive at the resting free Ca^{2+} concentration in synaptosomes (≈80 nM; ref. 18), whereas it is fully active at concentrations >400 nM. This may constitute the physiological mechanism for stimulating the synthesis of NO, which is also Ca^{2+}-dependent in cerebellar cells (13). Interestingly, the guanylate cyclase was inhibited by physiological Ca^{2+} concentrations (confirming a previous report; ref. 19). This would minimize changes in guanylate cyclase activity in cells stimulated to produce NO and therefore would represent a control mechanism by which activated cells release NO to stimulate the guanylate cyclase selectively in target cells.

The mechanism by which L-arginine is oxidized to citrulline and NO is not known. Studies in the macrophage led to the suggestion that the reaction could consist of arginine deimination followed by oxidation of NH_3 to NO (5). However, we did not observe any synthesis of NO when the synaptosomal preparation was incubated with 200 μM NH_4Cl. Furthermore, N^{α}-benzoyl-L-arginine ethyl ester, a substrate for peptidyl-arginine deiminase (20), was not a substrate, and formamidine, an inhibitor of bacterial arginine deiminase (21), had no effect in the present study. On the basis of its NADPH dependence, we have suggested that the reaction in vascular endothelial cells is an oxygenation (9). A more detailed mechanistic proposal has also come from recent work on the macrophage enzyme suggesting the existence of a monooxygenation reaction followed by the formation of NO and citrulline by hydrolysis (6).

The enzymes in brain, macrophages, and endothelial cells that form NO from L-arginine share a number of characteristics: they are soluble, NADPH-dependent, and inhibited in an enantiomerically specific manner by L-MeArg (6, 9). It is highly likely that the enzyme in the neutrophil, where we have recently described the formation of NO from L-arginine (8), shares these same characteristics. There are, however, some differences that are worth mentioning. The endothelial cell cannot utilize L-arginine methyl ester (2), unlike the brain enzyme or the macrophage (22), and L-canavanine inhibits NO formation in the macrophage (23) and the neutrophil (8) but not in the brain or in endothelial cells (2). Furthermore, the enzyme in the macrophage requires Mg^{2+} for maximal activation (6). Whether some of these differences are due to differences in the enzyme or in the uptake mechanisms requires clarification. It is, however, likely that these tissues contain distinct isoenzymes for the generation of NO from L-arginine.

Our results provide an explanation for the observations of Deguchi and Yoshioka (10, 11), who described an arginine-dependent stimulation of the soluble guanylate cyclase in the central nervous system that exhibited the characteristics of the stimulation by a nitrovasodilator. It is clear that they were dealing with the L-arginine/NO pathway. Furthermore, our results also explain those of Garthwaite *et al.* (13), who described the release of an EDRF-like material from rat cerebellar cells stimulated with *N*-methyl-D-aspartate.

Our finding that the "EDRF" from the brain, like that from the vasculature, is NO further demonstrates the chemical nature of EDRF. Moreover, the occurrence of the L-arginine/NO pathway in the brain, vascular endothelium, and phagocytic cells indicates that this is a widespread transduction mechanism whose major function is the stimulation of the soluble guanylate cyclase.

The soluble guanylate cyclase in the brain is widely distributed and is stimulated by a number of mediators (24). It is therefore probable that in the central nervous system, as in other systems (25), the L-arginine/NO pathway plays a crucial regulatory role, the modulation of which leads to a variety of as yet undefined biological consequences.

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1. Palmer, R. M. J., Ferrige, A. G. & Moncada, S. (1987) *Nature (London)* **327**, 524–526.
2. Palmer, R. M. J., Ashton, D. S. & Moncada, S. (1988) *Nature (London)* **333**, 664–666.
3. Furchgott, R. F. & Zawadzki, J. V. (1980) *Nature (London)* **288**, 373–376.
4. Moncada, S., Radomski, M. W. & Palmer, R. M. J. (1988) *Biochem. Pharmacol.* **37**, 2495–2501.
5. Hibbs, J. B., Jr., Taintor, R. R. & Vavrin, Z. (1987) *Science* **235**, 473–476.
6. Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D. & Wishnock, J. S. (1988) *Biochemistry* **27**, 8706–8711.
7. Hibbs, J. B., Jr., Vavrin, Z. & Taintor, R. R. (1987) *J. Immunol.* **138**, 550–565.
8. McCall, T., Boughton-Smith, N. K., Palmer, R. M. J., Whittle, B. J. R. & Moncada, S. (1989) *Biochem. J.*, in press.
9. Palmer, R. M. J. & Moncada, S. (1989) *Biochem. Biophys. Res. Commun.* **158**, 348–352.
10. Deguchi, T. (1977) *J. Biol. Chem.* **252**, 7617–7619.
11. Deguchi, T. & Yoshioka, M. (1982) *J. Biol. Chem.* **257**, 10147–10151.
12. Miki, N., Kawabe, Y. & Kuriyama, K. (1977) *Biochem. Biophys. Res. Commun.* **75**, 851–856.
13. Garthwaite, J., Charles, S. L. & Chess-Williams, R. (1988) *Nature (London)* **336**, 385–388.
14. Patthy, A., Bajusz, S. & Patthy, L. (1977) *Acta Biochim. Biophys. Acad. Sci. Hung.* **12**, 191–196.
15. Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505.
16. Waldman, S. A. & Murad, F. (1987) *Pharmacol. Rev.* **39**, 163–196.
17. von Temkin, M. & Pyzhov, W. (1935) *Acta Physicochim. URSS* **2**, 473–486.
18. Ashley, R. H., Brammer, M. J. & Marchbanks, R. (1984) *Biochem. J.* **219**, 149–158.
19. Olson, D. R., Kon, C. & Breckenridge, B. M. (1976) *Life Sci.* **18**, 935–940.
20. Takahara, H., Oikawa, Y. & Sugawara, K. (1983) *J. Biochem.* **94**, 1945–1953.
21. Weickmann, J. L., Himmel, M. E., Smith, D. W. & Fahrney, D. E. (1978) *Biochem. Biophys. Res. Commun.* **83**, 107–113.
22. Iyengar, R., Stuehr, D. J. & Marletta, M. A. (1987) *Biochemistry* **84**, 6369–6373.
23. Iyengar, R., Stuehr, D. J. & Marletta, M. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6369–6373.
24. Drummond, G. I. (1984) in *Cyclic Nucleotides in the Central Nervous System*, ed. Drummond, G. I. (Raven, New York), pp. 40–125.
25. Moncada, S., Palmer, R. M. J. & Higgs, E. A. (1989) *Biochem. Pharmacol.* **38**, 1709–1715.