

Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum

(*N*-methyl-D-aspartate/arginine/citrulline/climbing fibers/inositol phospholipid cycle)

DAVID S. BRETT AND SOLOMON H. SNYDER*

Departments of Neuroscience, Pharmacology and Molecular Sciences, and Psychiatry and Behavioral Sciences, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205

Contributed by Solomon H. Snyder, August 18, 1989

ABSTRACT Nitric oxide, which mediates influences of numerous neurotransmitters and modulators on vascular smooth muscle and leukocytes, can be formed in the brain from arginine by an enzymatic activity that stoichiometrically generates citrulline. We show that glutamate and related amino acids, such as *N*-methyl-D-aspartate, markedly stimulate arginine–citrulline transformation in cerebellar slices stoichiometrically with enhancement of cGMP levels. *N*^ω-monomethyl-L-arginine blocks the augmentation both of citrulline and cGMP with identical potencies. Arginine competitively reverses both effects of *N*^ω-monomethyl-L-arginine with the same potencies. Hemoglobin, which complexes nitric oxide, prevents the stimulation by *N*-methyl-D-aspartate of cGMP levels, and superoxide dismutase, which elevates nitric oxide levels, increases cGMP formation. These data establish that nitric oxide mediates the stimulation by glutamate of cGMP formation.

The striking inability of blood vessels to respond to vasodilating substances in the absence of an intact endothelium was resolved by the discovery of an “endothelium-derived relaxing factor” that subsequently was shown to be identical to nitric oxide (NO) (1–3). Besides endothelial cells, NO formation has been demonstrated in macrophages (4), and indirect evidence suggests the formation of an endothelium-derived relaxing factor in brain tissue (5). An enzymatic activity forming NO from arginine has been reported in endothelial cells (6), macrophages (4), neutrophils (7), and brain homogenates (8). Direct experimental evidence has established a function for NO in relaxing the smooth muscle of blood vessels (9) and in mediating the cytotoxic effects of macrophages and neutrophils (10). A role for NO in the brain has been elusive.

Glutamate, the major excitatory neurotransmitter in the brain, acts through several receptor subtypes, some of which directly open ion channels, whereas others stimulate the inositol phospholipid cycle (11, 12) and some stimulate the formation of cGMP (13). The enhancement of cGMP formation by glutamate is most prominent in the cerebellum (14) where Purkinje cells possess the highest levels of cGMP (14), guanylate cyclase (15), GMP-dependent protein kinase, and its protein substrate (16).

In the present study we show a striking enhancement by glutamate and related excitatory amino acids of the conversion of arginine to NO and the associated formation of citrulline. Moreover, we show that *N*^ω-monomethyl-L-arginine (MeArg), a potent inhibitor of the enzymatic transformation of arginine to NO, blocks glutamate-elicited cGMP formation, an effect that is specifically reversible by excess arginine. These findings establish NO as a “messenger molecule” mediating glutamate synaptic actions upon cGMP.

MATERIALS AND METHODS

Materials. [³H]Arginine (53 Ci/mmol; 1 Ci = 37 GBq) was obtained from DuPont/NEN. cGMP radioimmunoassay kits were obtained from Amersham. MeArg was obtained from Calbiochem. All other chemicals were obtained from Sigma. Hemoglobin was prepared from methemoglobin as described (17).

Determination of NO. NO was measured as its breakdown product, NO₂⁻. Ten cerebella were homogenized in 12 ml of 0.32 M sucrose/20 mM Hepes, pH 7.2/0.5 mM EDTA/1 mM dithiothreitol and centrifuged at 20,000 × *g* 15 min, and the supernatant was passed over a 0.75-ml column of Dowex AG50WX-8 (Na⁺ form) to remove endogenous arginine. Incubations were initiated by addition of 340 μl of homogenate to buffer containing (final concentrations) 2 mM NADPH, 0.45 mM Ca²⁺ (1 μM free calcium), 200 μM arginine, [³H]arginine (1 μCi/ml), and various concentrations of MeArg in a total volume of 400 μl. After a 45-min incubation at 37°C, [³H]citrulline was assayed in 150-μl aliquots of the incubation mixture after separation from [³H]arginine by cation-exchange chromatography as described below. NO₂⁻ concentration was determined by adding 250 μl of the incubation mixtures to 250 μl of Greiss reagent/5% (vol/vol) H₃PO₄/1% sulfanilic acid/0.1% *N*-(1-naphthyl)-ethylenediamine. The reaction of NO₂⁻ with this reagent produces a pink color, which was quantified at 554 nm against standards in the same buffer.

Preparation of Brain Slices. Cerebella from 10-day-old rats were cut at 0.4-mm intervals in both the sagittal and coronal planes using a McIlwain tissue chopper. The slices were dispersed in Krebs–Henseleit buffer containing 118 mM NaCl, 4.7 mM KCl, 2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 11 mM glucose. Cerebellar slices from a single litter were pooled (≈10 rats) and were incubated for 60 min in 250 ml of buffer continuously gassed with 95% O₂/5% CO₂ at 37°C.

cGMP Levels and [³H]Citrulline Formation in Slices. After the 1-hr preincubation, 20-μl aliquots of gravity-packed slices were transferred to prewarmed 5-ml minivials (Beckman) containing 250 μl of Krebs–Henseleit buffer (equilibrated with 95% O₂/5% CO₂). Appropriate concentrations of MeArg were added and all slices were further incubated 20 min under 95% O₂/5% CO₂ at 37°C. For determinations of cGMP, after a 3-min exposure to excitatory amino acids, slices were inactivated by boiling for 5 min in 1 ml of 50 mM Tris·HCl, pH 8.4/5 mM EDTA. After sonication, cGMP levels were determined by radioimmunoassay. [³H]Citrulline accumulation after a 15-min exposure of slices to [³H]arginine (3 μCi/ml) and the appropriate excitatory amino acid was assessed by adding 0.75 ml of ice-cold buffer with 5 mM

arginine and 4 mM EDTA, whereupon the slices were centrifuged $10,000 \times g$ for 1 min at 0°C , the medium was removed, and 1 ml of 1 M trichloroacetic acid was added to precipitate protein. After sonication and centrifugation, the supernatants were removed and the trichloroacetic acid was extracted three times with 2 ml of diethyl ether. Aliquots of 0.5 ml of the extract were neutralized with 2 ml of 20 mM Hepes (pH 6.0) and applied to 2-ml columns of Dowex AG50WX-8 (Na^+ form) and material was eluted with 2 ml of water. ^3H Citrulline was quantified by liquid scintillation spectroscopy of the 4-ml flow-through, whose sole radioactive component was verified as ^3H citrulline by thin layer chromatography (data not shown).

RESULTS

Prior to initiating experiments with cerebellar slices, we examined the conversion of ^3H arginine to ^3H citrulline in cerebellar homogenates. As has been reported (8), we found this conversion to be completely dependent on added NADPH as well as added Ca^{2+} (data not shown). Fifty percent of maximal ^3H citrulline formation was apparent at about 150 nM calcium. We also have confirmed the kinetic constants for the arginine to citrulline conversion (8). We examined the conversion of increasing concentrations of ^3H arginine to ^3H citrulline and observed a K_m of $6 \mu\text{M}$ and V_{max} of 150 pmol per min per mg of protein. We also have examined the effects of various concentrations of MeArg on ^3H citrulline formation in cerebellar homogenates and observed a K_i for inhibition of ^3H citrulline formation of about $2 \mu\text{M}$ (data not shown). The conversion of ^3H arginine to ^3H citrulline appears to be stoichiometric with the formation of NO. MeArg displays purely competitive inhibition of both activities (Table 1).

NO is extraordinarily unstable with a half-life at room temperature of about 10 sec. (3). Most studies of NO formation have measured chemiluminescence of the complex formed between NO and ozone, which is a complex laborious procedure (2). We have developed a simple and sensitive procedure for monitoring the conversion of ^3H arginine to ^3H citrulline in brain slices (Fig. 1). This conversion is dramatically enhanced by glutamate and *N*-methyl-D-aspartate (NMDA). NMDA is the most potent agent examined, producing a 2.5-fold elevation in ^3H citrulline formation at $100 \mu\text{M}$ concentration and a maximal elevation of almost 3-fold at 1 mM concentration, as 5 mM NMDA produces no greater effect (data not shown). Glutamate is less potent than NMDA but produces the same maximal effect, while kainate

Table 1. Stoichiometric inhibition of ^3H citrulline and NO formation by MeArg

MeArg, μM	^3H Citrulline formation, % maximum	NO formation, % maximum	Calculated activity, % maximum
0	100	100	100
10	83	82	81
20	73	72	70
50	53	51	49
100	32	29	33
200	23	21	20
2000	0	0	2

^3H Citrulline and NO, measured as its breakdown product NO_2^- , were assayed in cerebellar homogenates with $200 \mu\text{M}$ ^3H arginine added. Calculated activities were determined assuming competitive inhibition by MeArg and the observed kinetic parameters, $K_m = 6 \mu\text{M}$ for arginine and $K_i = 1.5 \mu\text{M}$ for MeArg. Maximal levels of ^3H citrulline and NO formation were 10,200 cpm and $7.5 \mu\text{M}$, respectively. Data represent the mean of triplicate determinations and varied $<8\%$.

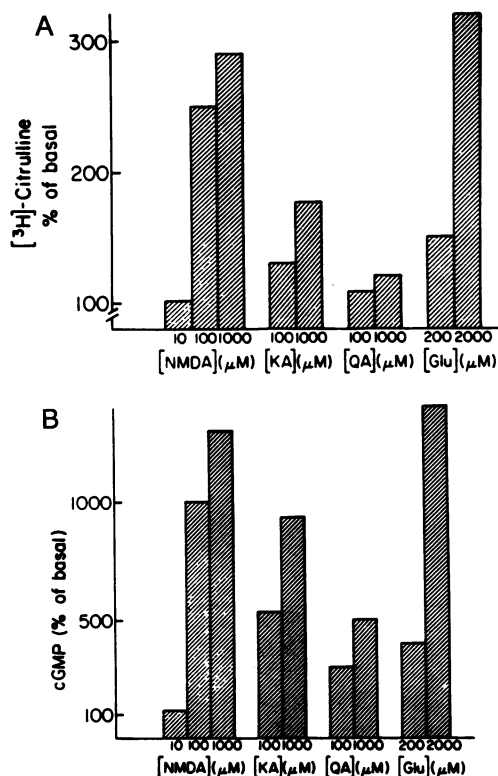


FIG. 1. Pharmacology of excitatory amino acid-mediated enhancement of ^3H arginine to ^3H citrulline conversion (A) and cGMP levels in cerebellar slices (B). Basal levels for ^3H citrulline formation were 1640 cpm and for cGMP levels were 2.4 pmol/mg of protein. Values are the mean of triplicate determinations with a SD $<15\%$. The experiments were repeated with similar results.

produces lower enhancement with only a 1.8-fold increase in ^3H citrulline at 1 mM kainate. Quisqualate at 0.1 and 1.0 mM has minimal effect on ^3H citrulline formation.

The relative potencies of glutamate and its derivatives in stimulating cGMP formation are the same as stimulating ^3H citrulline formation, with NMDA being most potent and glutamate somewhat less potent though producing the same maximal effect. Kainate produces less of a maximal stimulation of cGMP than NMDA or glutamate, and quisqualate is least active (Fig. 1B).

To determine whether the NMDA stimulation of NO formation and cGMP levels are linked, we compared the concentration-response effects of NMDA on ^3H citrulline and cGMP formation (Fig. 2A). Concentration-response curves for the two are virtually superimposable with negligible effects at $10 \mu\text{M}$ NMDA, marked effects at $100 \mu\text{M}$ NMDA, and maximal effects at 1 and 5 mM NMDA.

MeArg is a potent inhibitor of the conversion of arginine to NO with an IC_{50} in brain homogenates of about $5 \mu\text{M}$ (8). We compared the effects of MeArg on the NMDA enhancement of cGMP and ^3H citrulline formation in slices (Fig. 2B). MeArg displays identical potencies in blocking NMDA enhancement of cGMP and ^3H citrulline formation with an IC_{50} of about $6 \mu\text{M}$.

If MeArg blocks NMDA stimulation of cGMP formation by competitively inhibiting the conversion of arginine to NO, its influence should be reversed by arginine. Accordingly, we examined effects of increasing arginine concentrations on the ability of MeArg to block NMDA stimulation of cGMP formation (Fig. 3). In the absence of MeArg no enhancement of NMDA stimulation of cGMP levels occurs with 1–1000 μM arginine. Both $20 \mu\text{M}$ and $200 \mu\text{M}$ MeArg concentrations completely block NMDA stimulation of cGMP levels. Argi-

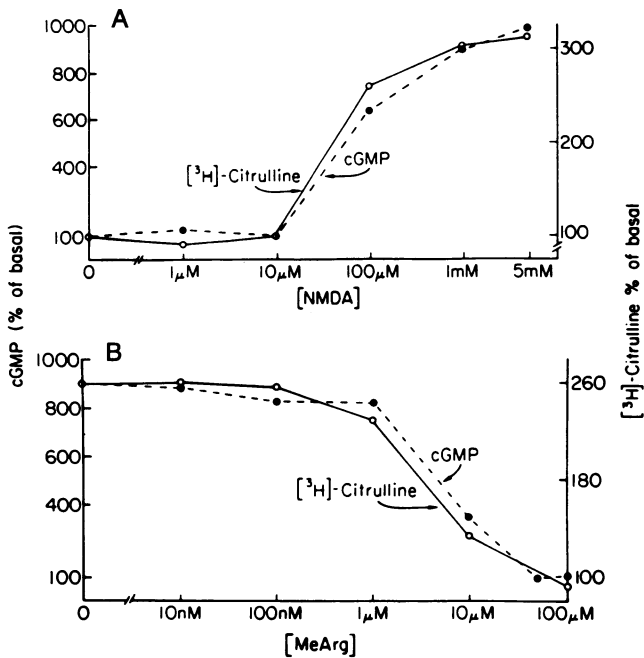


FIG. 2. Concentration-response relationships for NMDA stimulation (A) and MeArg inhibition (B) of [³H]citrulline and cGMP formation in cerebellar slices. All slices in B were stimulated with 500 μM NMDA. Basal levels for [³H]citrulline formation were 1478 cpm and for cGMP levels were 2.7 pmol/mg of protein. Data are from representative experiments in triplicate with SD <15% and were replicated twice.

nine reverses the effects of MeArg competitively. Thus 20 μM arginine provides a 50% reversal of the effects of 20 μM MeArg, and about 200 μM arginine is required to reverse by 50% the influences of 200 μM MeArg.

The competitive reversal by arginine of MeArg effects strongly suggests that MeArg actions on NMDA stimulation of cGMP levels derive from competitive inhibition of endogenous arginine. To provide further evidence for this specificity, we examined the ability of other amino acids to reverse the effects of MeArg (Fig. 4). We evaluated leucine, lysine, ornithine, citrulline, and alanine at 1 mM concentrations. None of these amino acids are able to reverse the MeArg blockade of NMDA-stimulated cGMP formation.

To obtain more direct evidence that arginine influences on cGMP derive from the formation of NO, we examined the

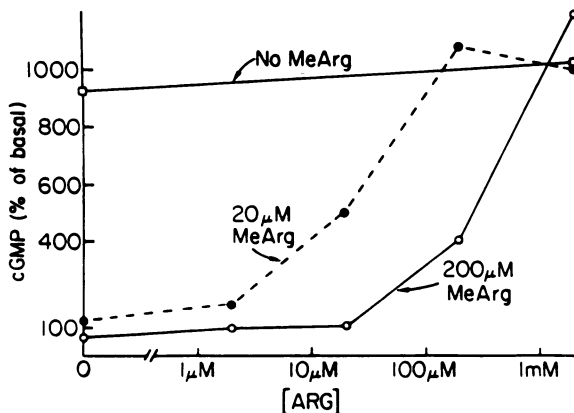


FIG. 3. Reversal of MeArg inhibition of NMDA-mediated enhancement of cGMP. All slices were stimulated with 500 μM NMDA. Basal level of cGMP was 2.5 pmol/mg of protein. Data are the mean of triplicate determinations with an SD <15%. This experiment was repeated twice.

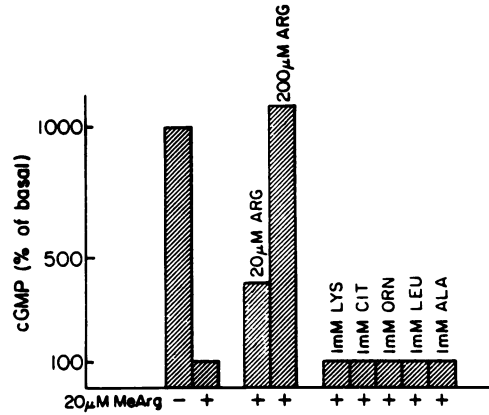


FIG. 4. Specificity of arginine reversal of MeArg inhibition of cGMP accumulation. All slices were treated with 500 μM NMDA. Basal level of cGMP was 3.1 pmol/mg of protein. All amino acids were added with MeArg 20 min prior to NMDA. Data are the mean of triplicate determinations from a representative experiment, which was repeated with similar events. +, MeArg added; -, no MeArg added; CIT, citrulline; ORN, ornithine.

effects of hemoglobin and superoxide dismutase (Fig. 5). Hemoglobin complexes with NO to block its stimulation of guanylate cyclase (18, 19), whereas superoxide dismutase enhances the effects of NO on guanylate cyclase by removing superoxides, which destroy NO. Hemoglobin prevents NMDA stimulation of cGMP levels with half-maximal effects at about 6 μM, consistent with the potency of hemoglobin in binding NO (18). Superoxide dismutase enhances the ability of 50 and 500 μM NMDA to stimulate cGMP levels.

DISCUSSION

To facilitate investigations of NO as a messenger substance, we have developed a simple and sensitive technique in which the conversion of [³H]arginine to [³H]citrulline is monitored, since the formation of citrulline is stoichiometric with the synthesis of NO. Utilizing this technique one can readily assay 100 samples in a day. The technique is highly sensitive and can easily detect as little as 1 pmol of [³H]citrulline formed in brain slices. By contrast, the formation of endogenous NO is most usually monitored by forming a complex of NO and ozone, which is then detected by chemilumines-

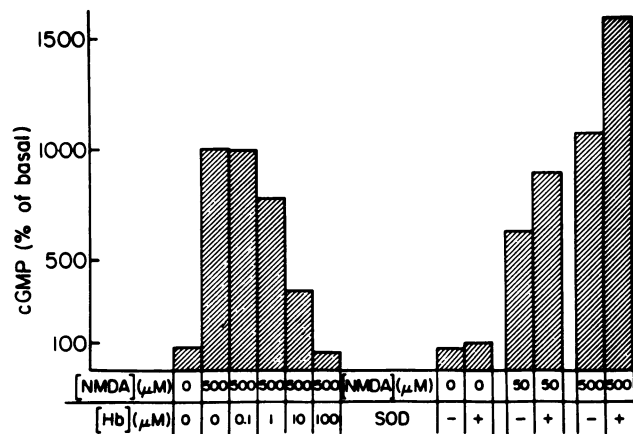


FIG. 5. Inhibition by hemoglobin (Hb) and potentiation by superoxide dismutase (SOD) of NMDA-mediated increase in cGMP in cerebellar slices. Hemoglobin and superoxide dismutase (100 units/ml) were added 5 min prior to NMDA. Basal level of cGMP was 2.7 pmol/mg of protein. Values are the mean of triplicate determinations with SD <15%. The experiment was repeated twice. +, Superoxide dismutase added; -, no Superoxide dismutase added.

cence. In this technique only one sample can be processed at a time, and nitroso derivatives are also detected.

We have obtained definitive evidence that NO mediates the stimulation by glutamate of cGMP levels in the cerebellum. Thus, the relative potencies of glutamate and several derivatives in stimulating the conversion of arginine to citrulline closely parallel their potencies in enhancing cGMP formation. The concentration-response curves for NMDA in elevating cGMP levels and stimulating citrulline formation are superimposable as are such curves for inhibition by MeArg of cGMP and citrulline formation. Inhibition by MeArg of cGMP formation is selectively reversed by arginine. Finally, hemoglobin, which potently complexes NO, inhibits NMDA stimulation of cGMP, and superoxide dismutase, which augments NO levels, enhances the formation of cGMP in response to NMDA.

The relative potencies of NMDA, glutamate, kainate, and quisqualate on [³H]citrulline and cGMP formation differ from any known excitatory amino acid receptor subtype (20). Since activation of climbing fibers dramatically augments cerebellar cGMP levels, while no known influence of parallel fibers has been described, it seems probable that Purkinje cell cGMP levels are most prominently regulated by climbing-fiber input. Aspartate is the putative transmitter of climbing fibers (21), suggesting that this unique receptor is selective for aspartate. In the absence of definitive evidence, it might best be designated the excitatory amino acid-cGMP (or EAA-cGMP) receptor. The identical amino acid specificity for [³H]citrulline and cGMP augmentation indicates that they involve the excitatory amino acid-cGMP receptor and supports a causal link between them.

What is the locus of cGMP in the cerebellum that is regulated by glutamate receptors? Immunohistochemical studies reveal a highly selective and pronounced concentration of cGMP-dependent protein kinase in Purkinje cells (16). Basal levels of cGMP are localized to Purkinje cells, since Purkinje cell-deficient mutant mice demonstrate a 70% depletion of cGMP (22). Immunohistochemical stains for guanylate cyclase localize this enzyme to Purkinje cells (15). Administration of harmaline to rats, which stimulates the firing of climbing fibers and the release of excitatory amino acid neurotransmitters at Purkinje cell synapses, markedly augments cerebellar levels of cGMP (23). The two excitatory inputs to Purkinje cells are climbing fibers and parallel fibers, the latter emanating from granule cells. Glutamate is thought to be the neurotransmitter of these parallel fibers. Parallel fibers can influence Purkinje cells by opening ion channels (24) or stimulating phosphatidyl inositol turnover (25), but it has not been established whether they can regulate cGMP levels. Although a substantial body of evidence suggests Purkinje cells as a site where cGMP levels are regulated, other evidence implies an involvement of granule cells and

glia (26). Experiments showing the release of a factor that relaxes smooth muscle and whose properties resemble NO imply the formation of NO by granule cells and stimulation of guanylate cyclase in glia (5).

This work was supported by United States Public Health Service Grant MH-18501, Research Scientist Award DA-00074 to S.H.S., Training Grant GM-07309 to D.S.B., and a gift from Bristol-Myers.

1. Moncada, S., Radomski, M. W. & Palmer, R. M. J. (1988) *Biochem. Pharm.* **37**, 2495-2501.
2. Furchgott, R. F. & Vanhoutte, P. M. (1989) *FASEB J.* **3**, 2007-2018.
3. Ignarro, L. J. (1989) *FASEB J.* **3**, 31-36.
4. Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D. & Wishnok, J. S. (1988) *Biochemistry* **27**, 8706-8711.
5. Garthwaite, J., Charles, S. J. & Chess-Williams, R. (1988) *Nature (London)* **336**, 385-388.
6. Palmer, R. M. T. & Moncada, S. (1989) *Biochem. Biophys. Res. Commun.* **158**, 348-352.
7. McCall, T., Boughton-Smith, N. K., Palmer, R. M. J., Whittle, B. J. R. & Moncada, S. (1989) *Biochem. J.*, in press.
8. Knowles, R. G., Palacios, M., Palmer, R. M. J. & Moncada, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5159-5162.
9. Gruetter, C. A., Gruetter, D. Y., Lyon, J. E., Kadonitz, P. T. & Ignarro, L. J. (1981) *J. Pharmacol. Exp. Ther.* **219**, 181-186.
10. Hibbs, J. B., Jr., Vavrin, Z. & Taintor, R. R. (1987) *J. Immunol.* **138**, 550-565.
11. Sladeczek, F., Recasens, M. & Bockaert, J. (1988) *Trends Neurosci.* **11**, 545-549.
12. Nicoletti, F., Wroblewski, J. T., Novelli, A., Alho, H., Guidotti, A. & Costa, E. (1986) *J. Neurosci.* **6**, 1905-1911.
13. Ferrendelli, J. A., Chang, M. M. & Kinscherf, D. A. (1974) *J. Neurochem.* **22**, 535-540.
14. Ferrendelli, J. A. (1978) *Adv. Cyclic Nucleotide Res.* **9**, 453-464.
15. Nahare, M., Ichihawa, M. & Deguchi, T. (1983) *Brain Res.* **273**, 9-15.
16. Nairn, A. C., Hemmings, H. C. & Greengard, P. (1985) *Annu. Rev. Biochem.* **54**, 931-976.
17. Martin, W., Villani, G. M., Jothianandan, D. & Furchgott, R. F. (1985) *J. Pharmacol. Exp. Ther.* **232**, 708-716.
18. Gibson, Q. H. & Houghton, F. J. W. (1957) *J. Physiol. (London)* **136**, 507-526.
19. Mittal, C. K., Arnold, W. P. & Murad, F. (1978) *J. Biol. Chem.* **253**, 1266-1271.
20. Monaghan, D. T., Bridges, R. J. & Cotman, C. W. (1989) *Annu. Rev. Pharmacol. Toxicol.* **29**, 365-402.
21. Ito, M. (1987) *Physiol. Bohemoslov.* **36**, 203-216.
22. Mao, C. C., Guidotti, A. & Landis, S. (1975) *Brain Res.* **90**, 335-339.
23. Biggio, G., Brodie, B. B., Costa, E. & Guidotti, A. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3592-3596.
24. Ito, M. & Kane, M. (1982) *Neurosci. Lett.* **33**, 253-258.
25. Blackstone, C., Supattapone, S. & Snyder, S. H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4316-4320.
26. Garthwaite, G. & Garthwaite, J. (1987) *J. Neurochem.* **48**, 29-39.