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Neuronal NADPH diaphorase is a nitric oxide synthase

(endothelium-derived relaxing factor/arginine/citrilline/cGMP)

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ABSTRACT **NADPH diaphorase histochemistry selectively labels a number of discrete populations of neurons throughout the nervous system. This simple and robust technique has been used in a great many experimental and neuropathological studies; however, the function of this enzyme has remained a matter of speculation. We, therefore, undertook to characterize this enzyme biochemically. With biochemical and immunochemical assays, NADPH diaphorase was purified to apparent homogeneity from rat brain by affinity chromatography and anion-exchange HPLC. Western (immunoblot) transfer and immunostaining with an antibody specific for NADPH diaphorase labeled a single protein of 150 kDa. Nitric oxide synthase was recently shown to be a 150-kDa, NADPH-dependent enzyme in brain. It is responsible for the calcium/calmodulin-dependent synthesis of the guanylyl cyclase activator nitric oxide from L-arginine. We have found that nitric oxide synthase activity and NADPH diaphorase copurify to homogeneity and that both activities could be immunoprecipitated with an antibody recognizing neuronal NADPH diaphorase. Furthermore, nitric oxide synthase was competitively inhibited by the NADPH diaphorase substrate, nitro blue tetrazolium. Thus, neuronal NADPH diaphorase is a nitric oxide synthase, and NADPH diaphorase histochemistry, therefore, provides a specific histochemical marker for neurons producing nitric oxide.**

The NADPH diaphorase histochemical technique is based on the presence in certain neurons of an enzyme that can catalyze the NADPH-dependent conversion of a soluble tetrazolium salt to an insoluble, visible formazan (1, 2). This method has proven useful for the examination of select populations of neurons in both experimental studies and in human neuropathology. In particular, NADPH diaphorase has been shown to be a selective marker for forebrain neurons containing both somatostatin and neuropeptide Y (3) and for the ascending cholinergic reticular system in the mesopontine tegmentum (4). This method has, therefore, been used to examine these neurons in Huntington disease (5), Alzheimer disease (6, 7), progressive supranuclear palsy (8), and ischemia (9, 10). NADPH diaphorase-containing neurons appear relatively resistant to anoxia and excitotoxic damage (11–13), and those in the striatum are selectively spared in Huntington disease (5).

Although the NADPH diaphorase activity has been well defined histochemically (14), the function of this enzyme has remained a mystery. Previous attempts to characterize the enzyme biochemically have been hampered by lack of a specific assay (15, 16) because several proteins can exhibit NADPH-dependent diaphorase activity in brain homogenate (17). Therefore, we have used both a biochemical assay and an antibody that specifically recognizes neuronal NADPH diaphorase (18, 19) to monitor purification of this enzyme from rat brain. Because NADPH diaphorase is an NADPH-dependent enzyme, a purification protocol similar to that recently used for the NADPH-dependent brain enzyme, nitric oxide synthase, was attempted (20).

MATERIALS AND METHODS

Histochemistry. Young adult male Wistar rats were anesthetized with sodium pentobarbital and perfused with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS). Twenty-micron-thick cryostat sections were prepared and incubated in primary antibody (18, 19) diluted 1:200 in PBS/0.3% Triton X-100/2% normal goat serum for 48 hr at 4°C. The sections were then rinsed in PBS and incubated with Texas red-labeled goat anti-rabbit IgG (Jackson Immunoresearch) diluted 1:40 for 1 hr at room temperature. The sections were rinsed, mounted on slides, and examined and photographed with a fluorescence microscope. The coverslips were then removed, and the sections were treated at 37°C for 30 min with 50 mM Tris chloride, pH 8/0.2% Triton X-100/0.5 mM nitro blue tetrazolium (NBT)/1 mM β-NADPH for demonstration of NADPH diaphorase activity. The sections were then rephotographed under bright-field illumination.

Enzyme Assays. NADPH diaphorase activity was assayed by measuring the reduction of 0.5 mM NBT with 1 mM β-NADPH in 0.3 ml of 50 mM Tris chloride, pH 8.0, at 37°C for 8 min. The reaction was stopped with 0.3 ml of 100 mM sulfuric acid, and the absorbance of the formazan product was determined at its isobestic wavelength, 585 nm. Nitric oxide synthase was assayed by measuring the formation of [^{3}H]citrulline from 15 μM [^{3}H]arginine (57 Ci/mmol; 1 Ci = 37 GBq; DuPont/NEN) in the presence of 1 mM β-NADPH and calmodulin at 10 μg/ml, according to the method of Bredt and Snyder (20).

Enzyme Purification. Thirteen whole rat brains were homogenized in 5 vol of 50 mM Tris chloride, pH 7.4/1 mM EDTA/soybean trypsin inhibitor (10 mg/liter)/bacitracin (10 mg/liter)/aprotinin (10 mg/liter)/phenylmethanesulfonyl fluoride (100 mg/liter). The material was centrifuged at 30,000 × *g* for 30 min, then mixed with adenosine 2′,5′-diphosphate-agarose for 30 min at 4°C, and poured into a column. The column was washed and then eluted with 10 mM β-NADPH. The fractions containing nitric oxide synthase and NADPH diaphorase activities were then applied to a Protein Pak DEAE 5P HPLC anion-exchange column (Waters) and eluted with a linear 0–0.4 M NaCl gradient.

Immunoblotting. Fractions obtained after chromatography were electrophoresed on a 7.5% SDS/PAGE gel and electrophoretically blotted onto Immobilon (Millipore). The

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Abbreviation: NBT, nitro blue tetrazolium.

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membrane was incubated overnight in primary antibody (18, 19) or normal rabbit serum, diluted 1:1000, and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) and detected using 5-bromo-4-chloro-3-indolyl phosphate and NBT.

Immunoprecipitation. Rat brain was homogenized and centrifuged as described above, and 200-μl samples of the crude supernatant were incubated with 2 μl of primary antibody or normal rabbit antibody for 1 hr at 4°C. Protein G–Sepharose (100 μl of a 10% suspension in Tris/borate/saline; Pharmacia) was then added and incubated for 1 hr at 4°C. The samples were then centrifuged. Aliquots of the supernatants and the resuspended pellets were assayed for NADPH diaphorase and nitric oxide synthase activities, as described above.

RESULTS

Immunohistochemical staining of the rat brain with antibody recognizing neuronal NADPH diaphorase selectively stained neurons throughout the brain with a distribution identical to that seen with NADPH diaphorase histochemistry (18). In double-labeling experiments of forebrain and mesopontine sections, all neurons immunohistochemically labeled with this antibody were found to be NADPH diaphorase positive, and *vice versa* (Fig. 1). Thus, this antiserum provides an immunochemical marker for neuronal NADPH diaphorase.

Because NADPH diaphorase is an NADPH-dependent enzyme, we initially decided to use a protocol based on that recently used for the purification of the NADPH-dependent enzyme, nitric oxide synthase (20). NADPH diaphorase activity could be detected and immunoprecipitated from the soluble supernatant from whole rat brain and from fractions eluted with a NaCl gradient from a 20-ml DEAE anion-exchange column. The NADPH diaphorase peak eluted from this column bound to adenosine 2′,5′-diphosphate–agarose, but, unlike most proteins, the immunoreactive NADPH diaphorase activity did not elute from the affinity column

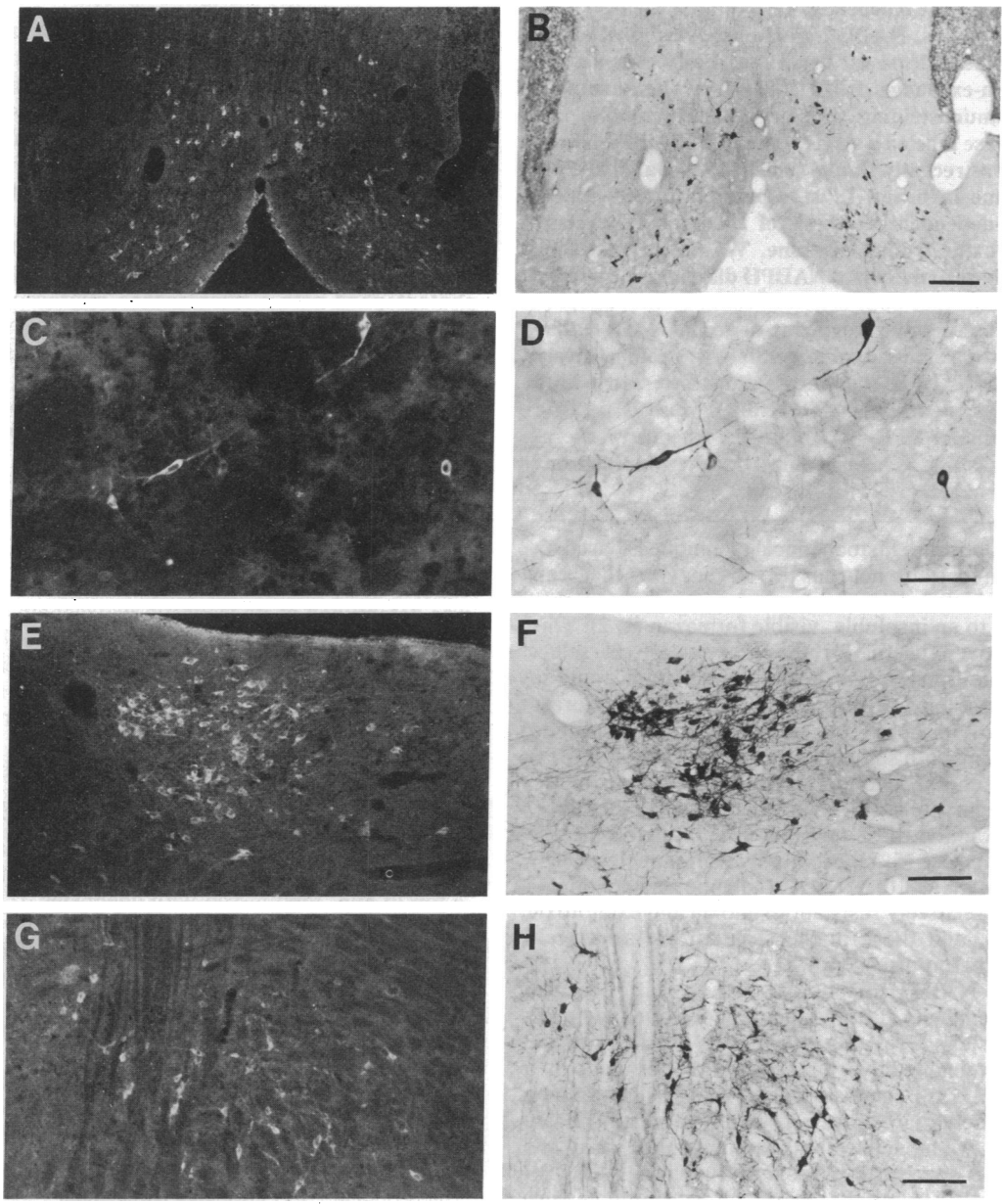

FIG. 1. Double staining of the rat brain using the indirect immunofluorescence procedure with the NADPH-diaphorase antibody (*A*, *C*, *E*, and *G*) followed by the NADPH diaphorase histochemical method on the same sections (*B*, *D*, *F*, and *H*). The two techniques label identical neuronal populations throughout the brain, including the magnocellular basal forebrain (*A* and *B*), the striatum (*C* and *D*), the laterodorsal tegmental nucleus (*E* and *F*), and the pedunculopontine tegmental nucleus (*G* and *H*).

with 0.5 M NaCl. Some NADPH diaphorase activity did elute in the salt wash; however, this activity was not recognized by the NADPH diaphorase-specific antibody and, therefore, does not correspond to the neuronal NADPH diaphorase detected histochemically. Immunoreactive NADPH diaphorase activity could, however, be subsequently eluted, with 10 mM β-NADPH. This chromatographic profile is similar to that reported for NADPH-dependent nitric oxide synthase using this same purification protocol (20), suggesting that a nitric oxide synthase might be responsible for the neuronal NADPH diaphorase activity.

The possibility that neuronal NADPH diaphorase and nitric oxide synthase were the same protein was investigated by attempting to copurify both enzyme activities. To speed up the purification and maintain the unstable nitric oxide synthase activity (20), we chose to use affinity chromatography followed by anion-exchange HPLC (Fig. 2). Nitric oxide synthase and NADPH diaphorase activities were found to copurify with this method, which yielded a single silver-stained protein band of 150 kDa apparent molecular mass on SDS/PAGE. This size is consistent with the size of nitric oxide synthase purified from rat cerebellum (20). This purified protein was specifically detected in immunoblotting experiments with the NADPH diaphorase-specific antiserum (Fig. 3).

Both nitric oxide synthase and NADPH diaphorase activities could be immunoprecipitated from crude supernatants with the NADPH diaphorase-specific antiserum but not with nonimmune rabbit serum. The activities of both enzymes in

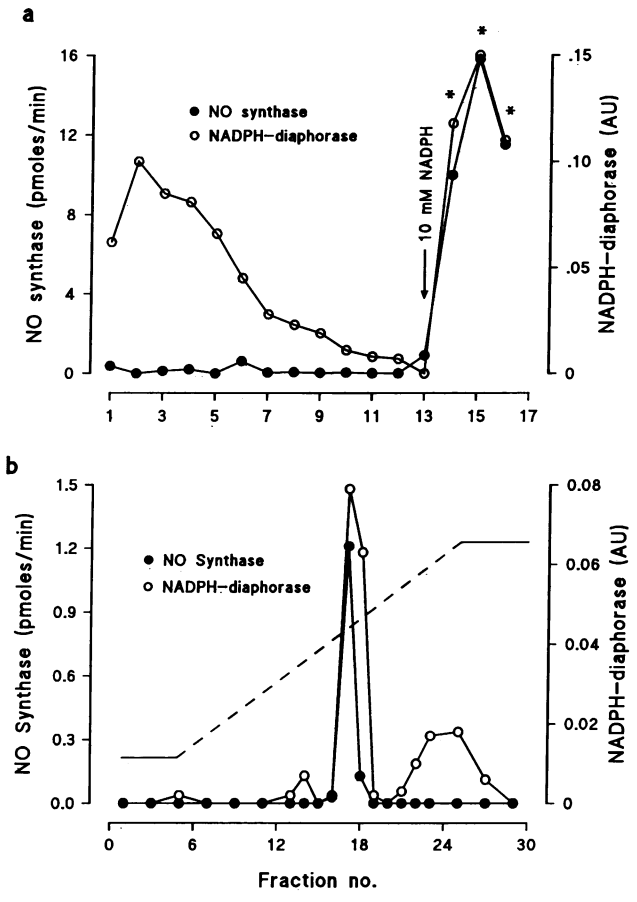

FIG. 2. Copurification of nitric oxide (NO) synthase and NADPH diaphorase activities from rat brain. Fractions containing nitric oxide synthase and NADPH diaphorase activities eluted from an adenosine 2′,5′-diphosphate–agarose affinity column with 10 mM β-NADPH (*) (*a*) were subsequently run on DEAE anion-exchange HPLC and eluted with 0–0.4 M NaCl gradient (*b*).

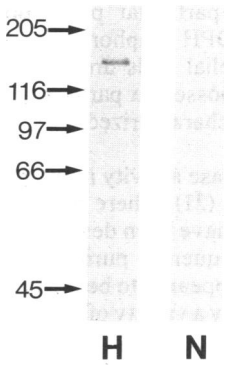

FIG. 3. Immunoblot of the fractions containing nitric oxide synthase and NADPH diaphorase activities after affinity chromatography and anion-exchange HPLC. A single protein of 150-kDa apparent molecular mass is recognized by the NADPH-diaphorase antiserum (H), but not by normal rabbit serum (N).

the supernatant were reduced by 10–15% after immunoprecipitation. The pellet with the NADPH diaphorase-specific antibody showed a positive NADPH diaphorase reaction and formed 4 pmol of citrilline per hour, whereas the pellet with normal rabbit serum produced blank values for both assays.

Nitric oxide synthase requires NADPH as a cofactor for conversion of L-arginine to citrulline and nitric oxide (20–22). If nitric oxide synthase is NADPH diaphorase then NBT, the substrate for the NADPH diaphorase histochemical reaction, should be able to compete with L-arginine for reducing equivalents from NADPH. We found that NBT inhibited nitric oxide synthase activity competitively, with respect to arginine, with a K_i of 11 μM (Fig. 4). The K_m of the nitric oxide synthase for arginine was found to be 2.9 μM, which is similar to reports (21–22) for the enzyme from forebrain and cerebellum.

DISCUSSION

Pharmacological experiments with a number of substrates, cofactors, and inhibitors indicate that distinct nitric oxide synthase isoenzymes are present in various tissues. The macrophage and forebrain forms can use L-arginine methyl ester as a substrate (21, 23), whereas the endothelial form cannot (24). The macrophage form is inhibited by L-canavanine (23), whereas the neuronal and endothelial enzymes are

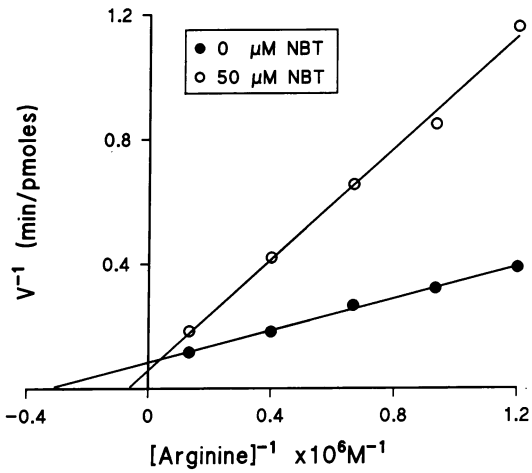

FIG. 4. Inhibition of nitric oxide synthase activity in a crude supernatant from whole rat brain by NBT. The activity (velocity, V) was measured at various substrate concentrations with or without added inhibitor.

not (21, 24). Only particular populations of neurons are stained by the NADPH diaphorase technique, and macrophages and endothelial cells are not stained. Thus, some neurons appear to possess a particular form of nitric oxide synthase, which is characterized by strong NADPH diaphorase activity.

Nitric oxide synthase activity in the rat brain was detected in the forebrain first (21), where many NADPH diaphorase-containing neurons have been described (1, 2). A nitric oxide synthase was subsequently purified from cerebellum (20), where the enzyme appeared to be present in granule cells (25) and to be activated by a variety of stimuli including *N*-methyl-D-aspartate receptor agonists (22, 26). In the cerebellar cortex the granule cells show NADPH diaphorase activity; however, the reaction is not strong, and the granule cells are not immunohistochemically stained by using the NADPH diaphorase-specific antibody. This result suggests that the cerebellum may contain a distinct isoform of the 150-kDa nitric oxide synthase, perhaps corresponding to the endothelial enzyme type (27). Indeed, there is pharmacological evidence for two nitric oxide synthase isozymes in cerebellum (28).

Nitric oxide synthase can produce nitric oxide in an NADPH-dependent fashion in response to changes in intracellular free calcium by deimidating arginine to citrulline (21). The selective coexistence of citrulline-like immunoreactivity in NADPH diaphorase-positive neurons (S.R.V., B.T.V., and B. Pasqualotto, unpublished observations) is thus consistent with NADPH diaphorase being a nitric oxide synthase. Nitric oxide is membrane permeable and may, therefore, have effects in surrounding cells, as well as in the cells in which it is formed. Nitric oxide appears to act by stimulating soluble guanylyl cyclase (29, 30) and may also directly activate a cytoplasmic protein ADP-ribosyltransferase (31). Nitric oxide has been suggested as a possible factor released from postsynaptic components to facilitate presynaptic release in surrounding synapses during long-term potentiation (26). Nitric oxide may also be released from nerve terminals in response to depolarization in a neurotransmitter-like fashion, to act upon postsynaptic cells (32). In addition, nitric oxide is well suited to regulate local cerebral blood flow in response to neuronal activity. Our data indicate that NADPH diaphorase is a neuronal nitric oxide synthase. Thus, the extensive literature on the histochemistry of NADPH diaphorase should be reexamined in light of the fact that this simple histochemical technique allows the cellular localization of nitric oxide synthase in the nervous system.

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1. Scherer-Singler, U., Vincent, S. R., Kimura, H. & McGeer, E. G. (1983) *J. Neurosci. Methods* **9**, 229–234.
2. Vincent, S. R. (1986) in *Neurohistochemistry: Modern Methods and Applications*, eds. Panula, P. Päivräinta, H. & Soinila, S. (Liss, New York), pp. 375–396.
3. Vincent, S. R., Johansson, O., Hökfelt, T., Skirboll, L., Elde, R. P., Terenius, L., Kimmel, J. & Goldstein, M. (1983) *J. Comp. Neurol.* **217**, 252–263.
4. Vincent, S. R., Satoh, K., Armstrong, D. M. & Fibiger, H. C. (1983) *Neurosci. Lett.* **43**, 31–36.
5. Ferrante, R. J., Kowall, N. W., Beal, M. F., Richardson, E. P., Jr., Bird, E. D. & Martin, J. B. (1985) *Science* **230**, 561–564.
6. Kowall, N. W. & Beal, M. F. (1988) *Ann. Neurol.* **23**, 105–114.
7. Mufson, E. J., Mash, D. C. & Hersh, L. B. (1988) *Ann. Neurol.* **24**, 623–629.
8. Hirsch, E. C., Graybiel, A. M., Duyckaerts, C. & Javoy-Agid, F. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5976–5980.
9. Ferriero, D. M., Arcavi, L. J., Sagar, S. M., McIntosh, T. K. & Simon, R. P. (1988) *Ann. Neurol.* **24**, 670–676.
10. Uemura, Y., Kowall, N. W. & Beal, M. F. (1990) *Ann. Neurol.* **27**, 620–625.
11. Beal, M. F., Kowall, N. W., Ellison, D. W., Mazurek, M. F., Swartz, K. J. & Martin, J. B. (1986) *Nature (London)* **321**, 168–171.
12. Koh, J.-Y., Peters, S. & Choi, D. W. (1986) *Science* **234**, 73–76.
13. Koh, J.-Y. & Choi, D. W. (1988) *J. Neurosci.* **8**, 2153–2163.
14. Hope, B. T. & Vincent, S. R. (1989) *J. Histochem. Cytochem.* **37**, 653–661.
15. Kemp, M. C., Kuonen, D. R., Sutton, A. & Roberts, P. J. (1988) *Biochem. Pharmacol.* **37**, 3063–3070.
16. Kuonen, D. R., Kemp, M. C. & Roberts, P. J. (1988) *J. Neurochem.* **50**, 1017–1025.
17. Levine, W. (1960) *J. Neurochem.* **6**, 28–35.
18. Michael, G. J., Joseph, S. A. & Knigge, K. M. (1989) *Soc. Neurosci. Abstr.* **15**, 561.
19. Knigge, K. M., Piekut, D. T., Abood, L. G., Joseph, S. A., Michael, G. J., Xin, L. & Berlove, D. J. (1989) *Methods Enzymol.* **178**, 212–221.
20. Bredt, D. S. & Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 682–685.
21. Knowles, R. G., Palacios, M., Palmer, R. M. J. & Moncada, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5159–5162.
22. Bredt, D. S. & Snyder, S. H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9030–9033.
23. Iyengar, R., Stuehr, D. J. & Marletta, M. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6369–6373.
24. Palmer, R. M. J., Ashton, D. S. & Moncada, S. (1988) *Nature (London)* **333**, 664–666.
25. Garthwaite, J. & Garthwaite, G. (1987) *J. Neurochem.* **48**, 29–39.
26. Garthwaite, J., Charles, S. L. & Chess-Williams, R. (1988) *Nature (London)* **336**, 385–388.
27. Ross, C. A., Bredt, D. & Snyder, S. H. (1990) *Trends Neuro-Sci.* **13**, 216–222.
28. East, S. J. & Garthwaite, J. (1990) *Eur. J. Pharmacol.* **184**, 311–313.
29. Arnold, W. P., Mittal, C. K., Katsuki, S. & Murad, F. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3203–3207.
30. Miki, N., Kawabe, Y. & Kuryama, K. (1977) *Biochem. Biophys. Res. Commun.* **75**, 851–856.
31. Brüne, B. & Lapetina, E. G. (1989) *J. Biol. Chem.* **264**, 8455–8458.
32. Bult, H., Boeckxstaens, G. E., Pelckmans, P. A., Jordaens, F. H., Van Maercke, Y. M. & Herman, A. G. (1990) *Nature (London)* **345**, 346–347.