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Hydrogen Sulfide Induces Cyclic AMP and Modulates the NMDA Receptor

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Hydrogen sulfide (H_2S) is produced endogenously from L-cysteine in mammalian tissues, and may function as a neuromodulator in the brain as well as a tone regulator in smooth muscle. H_2S is present at relatively high levels in the brain, and cystathionine β-synthase (CBS), which is highly expressed in the hippocampus, is involved in the production of brain H_2S. Physiological concentrations of H_2S selectively enhance NMDA receptor-mediated currents and facilitate the induction of hippocampal long-term potentiation (LTP). The NMDA receptor subunits are directly phosphorylated at specific sites by protein kinase A (PKA), resulting in the activation of NMDA-receptor-mediated excitatory postsynaptic currents. PKA activation is also observed in the induction of LTP. Here we show that physiological concentrations of H_2S increase the production of cAMP in primary cultures of brain cells, neuronal and glial cell lines, and *Xenopus* oocytes. NMDA receptors expressed on *Xenopus* oocyte membrane are modulated by H_2S. This modulation by H_2S is specifically inhibited by adenylyl cyclase-specific inhibitor MDL-12,330A. The present findings provide a mechanism for the previous observation that H_2S modulates NMDA receptors and enhances the induction of LTP. © 2000 Academic Press

Key Words: **hydrogen sulfide; gaseous neuromodulator; cAMP; NMDA receptor.**

Endogenous hydrogen sulfide (H_2S) can be formed from cysteine by pyridoxal-5′-phosphate-dependent enzymes, including cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (1–4). Endogenous levels of H_2S in the brain have been measured in the rat, human, and bovine (5–7). The relatively high concentration (50–160 μM) of endogenous H_2S in the brain suggests that it has a physiological function. CBS is highly expressed in the hippocampus and cerebellum, and brain homogenates produce H_2S in the presence of cysteine and pyridoxal-5′-phosphate (8). The production of H_2S is inhibited by CBS inhibitors and is increased by an activator of CBS, indicating that CBS contributes to the production of endogenous H_2S. Although high concentrations of H_2S inhibit synaptic responses, physiological concentrations of H_2S facilitate the induction of LTP in the hippocampus (8). Another H_2S producing enzyme, CSE, is expressed in the ileum, portal vein, and thoracic aorta (9). These tissues produce H_2S, and H_2S relaxes these smooth muscles in synergy with NO (9). These observations suggest that endogenous H_2S functions as a neuromodulator in the brain as well as a tone regulator in smooth muscle.

Cyclic-AMP (c-AMP)-dependent protein kinase (PKA) is a possible regulator of the increase in synaptic strength during long-term potentiation (LTP). LTP in the CA1 region is completely blocked when a PKA inhibitor is injected postsynaptically (10), and PKA activation has been observed at the initiation of LTP as well as the late phase of LTP (11, 12). The activation of the NMDA receptor is required for the induction of LTP (13, 14). The NMDA receptor subunits NMDAR1, NMDAR2A and NMDAR2B are directly phosphorylated at specific sites by protein kinase A (PKA); the NMDAR1 subunit contains a consensus PKA phosphorylation site (15, 16). The increased activity of adenylyl cyclase and PKA enhances NMDA currents in dorsal horn (17), amygdala (18), and neostriatum (19), indicating that the NMDA receptor may be modulated through the cAMP cascade.

The present study shows that physiological concentrations of H_2S induced the production of cAMP in primary cultures of brain cells as well as neuronal and glial cell lines. H_2S modulates NMDA receptor ex-

Abbreviations used: H_2S, hydrogen sulfide; CBS, cystathionine β-synthase; NMDA, *N*-methyl-D-aspartate; LTP, long-term potentiation; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; IBMX, 3-isobutyl-1-methylxanthine; MDL-12,330A, *cis*-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine), HCl; cGMP, cyclic guanosine monophosphate.

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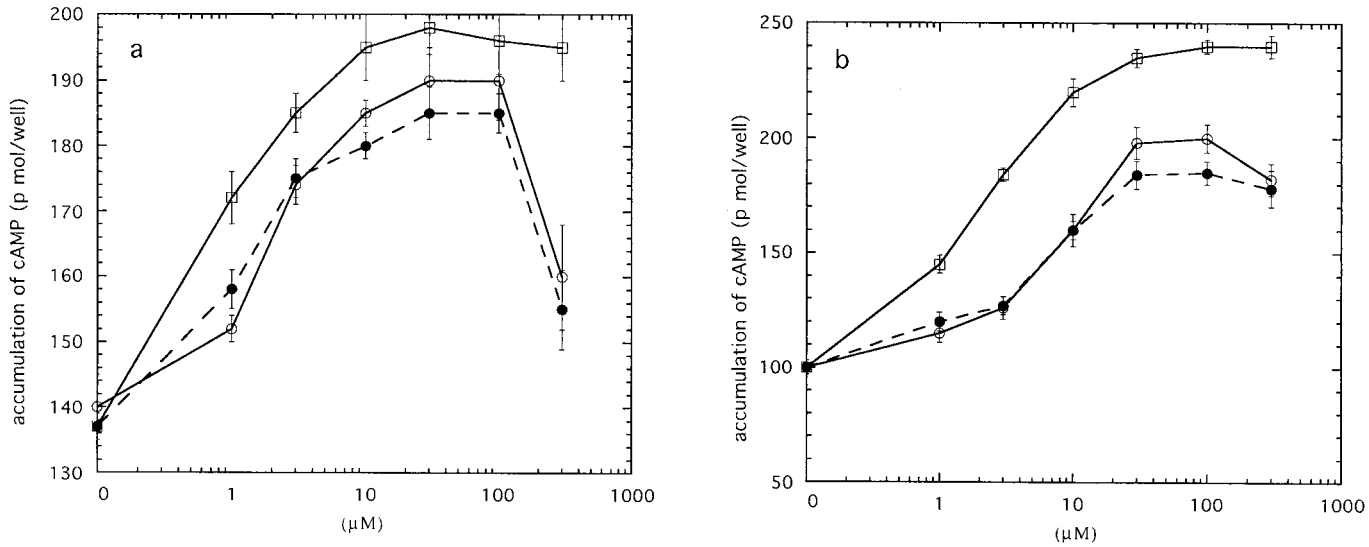

FIG. 1. Induction of cAMP in primary cultures of brain cells by NaHS. 3×10^3 cells/well of embryonic day 17 primary cultures of cerebral cortex (a) and cerebellum (b) were cultured in 96-well plates for 1 day (○) or 10 days (■). Cells were exposed to 100 μM IBMX for 10 min and then NaHS (○ and ■) was applied in the presence of IBMX and isoproterenol (□) for 5 min. Cells were lysed and the amounts of cAMP were measured by using cAMP EIA system (Amersham Pharmacia Biotech). Each experiment was repeated for four times and each value is shown as mean ± SE.

pressed in *Xenopus* oocytes, and this activation is blocked by an adenylyl cyclase specific inhibitor. These observations suggest that H_2S modulates NMDA receptors by inducing the production of cAMP.

EXPERIMENTAL PROCEDURES

NaHS was purchased from Aldrich (Wisconsin), and MDL-12,330A, KT5720, myristoylated PKA inhibitor (14–22) amide were from Calbiochem (California). cAMP enzyme immunoassay (EIA) system was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK), and other chemicals were from Sigma (Missouri).

Cerebral and cerebellar neurons were obtained from 17-day-old embryos of Sprague-Dawley rats and enzymatically dissociated as described in a previous paper (20). The cells were suspended and plated on polylysine-coated 96-well microtiter dishes.

For the measurement of cAMP, 1×10^3 cells/well of neuronal cell lines and 3×10^3 cells/well of primary cultures of cerebral and cerebellar neurons were incubated for up to 10 days in DMEM supplemented with 10% fetal calf serum for neuronal cell lines and in DMEM containing 30 mM glucose, 2 mM glutamine, 1 mM pyruvate supplemented with 10% fetal calf serum for primary cultures of neurons. After the application of 100 μM IBMX in serum-free DMEM for 10 min, NaHS was applied for 5 min in the presence of 100 μM IBMX. Cells were lysed and amount of cAMP measured by a cAMP enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech).

Collagenase-treated oocytes were incubated in Barth's medium (88 mM NaCl, 1mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM Hepes, pH 7.4) for four days following the procedure described (21). Oocytes were exposed to 5 μM IBMX for 10 min, then NaHS applied in the presence of IBMX for 5 min in frog Ringer solution (120 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM Hepes, pH 7.4). Four oocytes in each group were lysed and the amounts of cAMP measured by using cAMP EIA system (Amersham Pharmacia Biotech).

For expression in *Xenopus* oocytes, plasmids containing NMDAR1 and NMDAR2A cDNAs obtained from Dr. Nakanishi were linearized with restriction enzymes and transcribed with T3 and T7 RNA polymerase. 50 nl of the resultant cRNA solutions (1 ng/nl) were injected into *Xenopus* oocytes, and the oocytes were incubated for four days in modified Barth's medium. Current recordings were performed in frog Ringer solution by using the two electrode voltage clamp system (Axoclamp 2A, Axon Instrument) as described (21). The membrane potential of oocytes was clamped at −55 mV.

For measurement of the onset time to respond to NMDA, the frog Ringer solution stained with methylene blue was used to check the time for frog Ringer solution flew from the valve to reach oocytes. The time was subtracted from the total time, and it was shown as the time required to respond to NMDA.

RESULTS AND DISCUSSION

Since cAMP regulates the activity of the NMDA receptor as well as the induction of LTP (11, 15–19, 22, 23), and since H_2S potentiates NMDA receptor-mediated currents and enhances the induction of LTP (8), it is possible that the effects of H_2S are mediated by cAMP. This possibility was examined by applying NaHS in the presence of 100 μM IBMX to primary cultures of rat cortical and cerebellar neurons. Primary cultures of neurons prepared from cerebral cortex and cerebellum responded to 1 μM NaHS and reached a maximum response at 30 μM (Fig. 1). Concentrations higher than 100 μM had less than maximal effectiveness (Fig. 1). Primary cultures of neurons incubated for 1 day and for 10 days respond equally well to NaHS (Fig. 1). The β-adrenergic agonist, isoproterenol, increases the production of cAMP in a dose-dependent

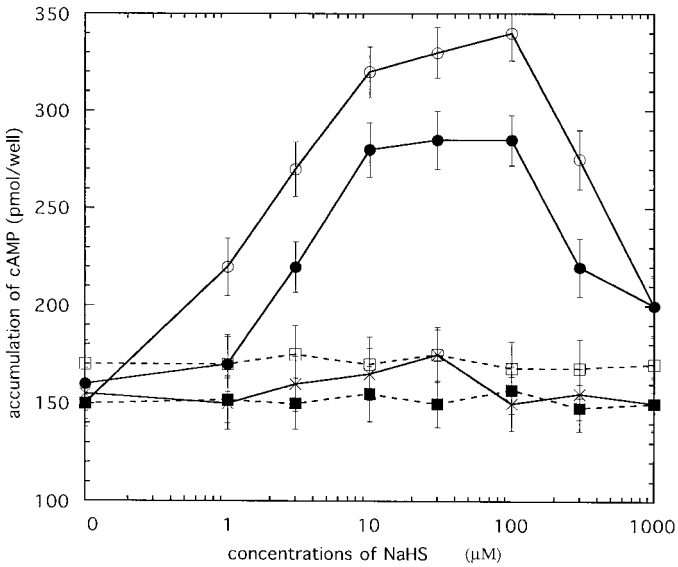

FIG. 2. Induction of cAMP in neuronal and glial cell lines by NaHS. 1×10^3 cells/well of B12 (○), B49 (X), B50 (●), B103 (□), and B104 (■) were cultured in 96-well plates for 24 hrs. Cells were exposed to 100 μM IBMX for 10 min and then NaHS was applied in the presence of IBMX for 5 min. Cells were lysed and the amounts of cAMP were measured by using cAMP EIA system (Amersham Pharmacia Biotech). Each experiment was repeated four times and each value is shown as mean ± SE.

manner, and the response is saturated at 30 μM (Fig. 1). These observations show that primary cultures of neurons respond to H_2S by the production of cAMP.

To determine which cell type in the brain responds to H_2S, the effect of H_2S on neuronal and glial cell lines established from the rat brain was tested (24). B50, B103 and B104 are nerve cells and produce regenerative action potentials, while B12 and B49 cells are glia (24). After the application of 100 μM IBMX for 10 min, NaHS was applied for 5 min in the presence of 100 μM IBMX. Cells were lysed and the amount of cAMP measured by a cAMP enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech). NaHS induced the production of cAMP, with a maximum induction at 10 μM in B12 and B50 cells. Concentrations higher than 100 μM were less efficient in the production of cAMP than 10 μM (Fig. 2). B49 also weakly responded to NaHS and produced cAMP. In contrast, NaHS did not induce cAMP in B103 and B104 (Fig. 2). These observations show that both neuronal and glial cells can respond to NaHS, and that cAMP can be produced as a second messenger for H_2S signal transduction in the responding cells.

Since the activation of adenylyl cyclase and PKA enhances NMDA receptor activity with the production of cAMP (17–19), and since H_2S induces the production of cAMP in the primary cultures of brain cells, it is possible that H_2S regulates the function of the NMDA receptor by inducing the production of cAMP. To address this problem, we examined the effect of H_2S on NMDA receptor expressed in *Xenopus* oocytes. Since H_2S induces the production of cAMP in primary cultures of CNS neurons as well as neuronal and glial cell lines, H_2S may also stimulate the production of cAMP in *Xenopus* oocytes. To examine this possibility, the effects of H_2S on the production of cAMP in the *Xenopus* oocytes were examined. Collagenase-treated oocytes were incubated in Barth's Medium for four days, and the oocytes were exposed to 5 μM IBMX for 10 min. Exposure of oocytes to NaHS in the presence of 5 μM IBMX for 5 min induced the production of cAMP in a dose-dependent manner, which reached to a maximum at 100 μM (Fig. 3). The production of cAMP was blocked by 200 μM MDL-12,330A, an adenylyl cyclase specific inhibitor (Fig. 3). These observations indicate that *Xenopus* oocytes respond to H_2S and produce cAMP, and allow us to assay NMDA receptor activity in the presence of H_2S.

Oocytes expressing NMDA receptors were exposed to 5 μM IBMX and NaHS for 5 min, and 100 μM NMDA with 10 μM glycine was applied and time required to respond to NMDA measured (Fig. 4a). NaHS decreased the time required to respond to NMDA in a dose-dependent manner (Fig. 4b). This effect of NaHS was

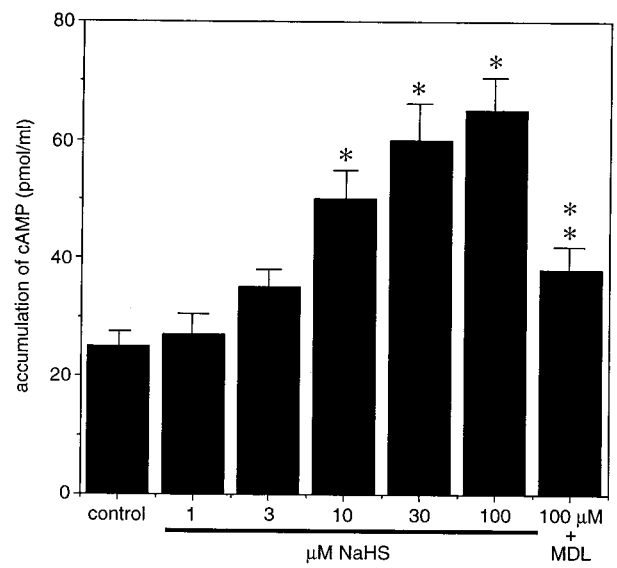

FIG. 3. Induction of cAMP in *Xenopus* oocytes by NaHS. Collagenase-treated oocytes were incubated in Barth's medium for four days following the procedure described (21). Oocytes were exposed to 5 μM IBMX for 10 min and then NaHS was applied in the presence of IBMX for 5 min. Four oocytes in each group were lysed with and the amounts of cAMP were measured by using cAMP EIA system (Amersham Pharmacia Biotech). Each experiment was repeated four times and each value was shown as mean ± SE. The values induced by NaHS are significantly different from those of control (*$P < 0.05$ by Student t-test). 200 μM MDL-12,330A significantly blocked the effect of 100 μM NaHS (**$P < 0.05$).

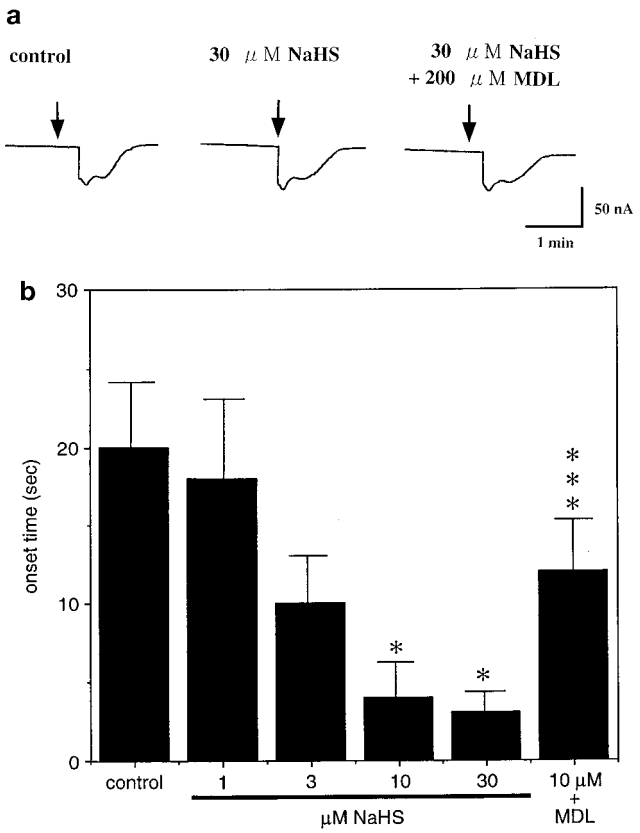

FIG. 4. The activation of NMDA receptor by NaHS. (a) Representative physiological responses of NMDA receptor expressed on *Xenopus* oocyte membrane. Oocytes were voltage clamped as described (Kimura and Schubert, 1993) and exposed to NaHS and 5 μM IBMX for 5 min. Then 100 μM NMDA with 10 μM glycine was applied to the oocytes at the time indicated by arrow, and the time required to respond to NMDA was measured. (b) Time required to respond to NMDA. Each experiment was repeated four times and each value is shown as mean ± SE. The values induced by NaHS are significantly different from those of control (*$P < 0.05$ by Student *t*-test). 200 μM MDL-12,330A significantly blocked the effect of 10 μM NaHS (***$P < 0.05$).

suppressed by 200 μM MDL-12,330A (Fig. 4b). Concentrations higher than 200 μM MDL-12,330A made the membrane potential of the *Xenopus* oocyte unstable, and were not used to test for the complete inhibition of the effect of NaHS. These observations suggest that H_2S increases the sensitivity of NMDA receptor to its ligand by inducing the production of cAMP.

Physiological concentrations of H_2S induce the production of cAMP in the primary cultures of CNS neurons as well as in the neuronal and glial cell lines. Both B12, cells of glial origin, and B50, excitable cells of neural origin respond to H_2S and produce cAMP. B49, another glial cell line, weakly responds to H_2S (Fig. 2). In contrast, B103 AND B104, excitable cells of neuronal origin do not respond to NaHS (Fig. 2). These observations suggest that some neuronal and glial cells may have receptors for H_2S which lead to the production of cAMP. Alternatively, the cells sensitive to H_2S may have a type of adenylyl cyclase which is activated directly by H_2S.

It is interesting to note that responses to isoproterenol are saturated at 10 μM, while concentrations of NaHS higher than 100 μM induce less cAMP than the lower concentrations. One possibility is that high concentrations of H_2S are toxic (8). Another possibility is that the receptor for H_2S, if any, may desensitize at higher concentrations.

The present observations that H_2S facilitates the activity of the NMDA receptor support our previous finding that H_2S enhances the NMDA receptor-mediated currents in the brain slices (8). An additional experiment to support the involvement of PKA in the activation of NMDA receptors by H_2S is to test whether the PKA inhibitors suppress the effect of H_2S. PKA specific inhibitors, KT 5720 and myristoylated PKA inhibitor (14–22) amide caused an instability in the resting membrane potential of *Xenopus* oocytes, making it difficult to examine the effect of these compounds. The above data show that in contrast to the other gaseous neurotransmitters/modulators, NO and CO, which activate guanylyl cyclase and increase the production of cGMP (25–26), H_2S has a unique signal transduction pathway. It is therefore likely that H_2S has a significant physiological function within the nervous system.

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REFERENCES

1. Stipanuk, M. H., and Beck, P. W. (1982) *Biochem. J.* **206,** 267–277.
2. Griffith, O. W. (1987) *in* Methods in Enzymology, Vol. 143, pp. 366–376. Academic Press, New York.
3. Erickson, P. F., Maxwell, I. H., Su, L. J., Baumann, M., and Glode, L. M. (1990) *Biochem. J.* **269,** 335–340.
4. Swaroop, M., Bradley, K., Ohura, T., Tahara, T., Roper, M. D., Rosenberg, L. E., and Kraus, J. P. (1992) *J. Biol. Chem.* **267,** 11455–11461.
5. Goodwin, L. R., Francom, D., Dieken, F. P., Taylor, J. D., Warenycia, M. W., Reiffenstein, R. J., and Dowling, G. (1989) *J. Anal. Toxicol.* **13,** 105–109.
6. Warenycia, M. W., Goodwin, L. R., Benishin, C. G., Reiffenstein, R. J., Francom, D. M., Taylor, J. D., and Dieken, F. P. (1989) *Biochem. Pharmacol.* **38,** 973–981.
7. Savage, J. C., and Gould, D. H. (1990) *J. Chromatogr.* **526,** 540–545.
8. Abe, K., and Kimura, H. (1996) *J. Neurosci.* **16,** 1066–1071.
9. Hosoki, R., Matsuki, N., and Kimura, H. (1997) *Biochem Biophys. Res. Commun.* **237,** 527–531.

10. Blitzer, R. D., Wong, T., Nouranifar, R., Iyengar, R., and Landau, E. M. (1995) *Neuron* **15,** 1403–1414.
11. Roberson, E. R. and Sweatt, J. D. (1996) *J. Biol. Chem*. **271,** 30436–30441.
12. Abel, T., Nguyen, P. V., Barad, M., Deuel, T. A. S., Kandel, E. R., and Bourctchouladze, R. (1997) *Cell* **88,** 615–626.
13. Collingridge, G. L., Kehl, S. J., and McLennan, H. (1983) *J. Physiol.* **334,** 33–46.
14. Harris, E. W., Ganong, A. H., and Cotman, C. W. (1984) *Brain Res.* **323,** 132–137.
15. Leonard, A. S., and Hell, J. W. (1997) *J. Biol. Chem*. **272,** 12107–12115.
16. Tingley, W. G., Ehlers, M. D., Kameyama, K., *et al.* (1997) *J. Biol. Chem*. **272,** 5157–5166.
17. Cerne, R., Rusin, K. I., and Randic, M. (1993) *Neurosci. Lett.* **161,** 124–128.
18. Huang, C. C., Tsai, J. J., and Gean, P. W. (1993) *Neurosci. Lett.* **161,** 207–210.
19. Colwell, C. S., and Levine, M. S. (1995) *J. Neurosci.* **15,** 1704–1713.
20. Abe, K., and Kimura, H. (1996) *J. Neurochem*. **67,** 2074–2078.
21. Kimura, H., and Schubert, D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7508–7512.
22. Frey, U., Huang, Y. Y., and Kandel, E. R. (1993) *Science* **260,** 1661–1664.
23. Matties, H., and Reymann, K. G. (1993) *NeuroReport* **4,** 712–714.
24. Schubert, D., Heinemann, S., Carlistle, W., *et al.* (1974) *Nature* **249,** 224–227.
26. Snyder, S. H., Jaffrey, S. R., and Zakhary, R. (1998) *Brain Res. Brain Res. Rev.* **26,** 167–175.