Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8301–8305, November 1988
Neurobiology

Purine metabolite inosine is an adrenergic neurotrophic substance for cultured chicken sympathetic neurons

(neuronal differentiation/adrenergic phenotypic expression/inosine/purine uptake)

ANNE D. ZURN*† AND KIM QUANG DO‡

*Department of Biochemistry, Sciences II, 30, quai E. Ansermet, CH-1211 Geneva 4, Switzerland; and ‡Brain Research Institute, Zurich University, August Forel Strasse 1, CH-8029 Zurich, Switzerland

Communicated by Edward Furshpan, July 12, 1988

ABSTRACT **Purines are ubiquitous endogenous cellular metabolites that have been postulated as neurotransmitters or neuromodulators in the nervous system. Recently, we showed that a low-molecular-mass component present in liver-conditioned medium selectively enhances the adrenergic properties of dissociated chicken sympathetic neurons in culture. We report here that this substance is inosine, a purine metabolite. Indeed, analysis of the low-molecular-mass fraction of liver-conditioned medium by HPLC shows that the neurotrophic activity coelutes with and has the same absorption spectrum as inosine. Inosine increases incorporation of [^{3}H]leucine into neuronal protein and stimulates catecholamine, but not acetylcholine, production by the sympathetic neurons in a dose-dependent fashion (half-maximal stimulation at 10^{-6} M). This effect can be blocked by 5×10^{-6} M dipyridamole, an inhibitor of nucleoside transport. Inosine therefore appears to be capable of modulating adrenergic phenotypic expression in cultured sympathetic neurons by acting via an as-yet-unknown intracellular pathway.**

A large body of evidence now exists supporting the idea that environmental signals regulate transmitter phenotypic expression (1). Modulation or induction of adrenergic properties occurs, for instance, in the presence of chicken embryo extract (2), neural tube-conditioned medium (3), nonneuronal cell-conditioned medium (4), and medium conditioned by embryonic chicken liver cells in culture (5, 6). So far, however, the biochemical nature of only the neurotrophic activity present in nonneuronal cell-conditioned medium (a 45-kDa glycoprotein) has been elucidated (7). Recently, we showed that the component in liver-conditioned medium (LCM) that increases neuronal growth and adrenergic, but not cholinergic, development in cultured chicken sympathetic neurons has a molecular mass <500 Da and is not inactivated by heat or Pronase treatment (6). We report here that this substance is inosine, the deaminated metabolite of adenosine.

Purines are ubiquitous endogenous cellular metabolites, which appear to be implicated in specific brain functions (8, 9). Adenosine, for instance, has a potent depressive effect on synaptic transmission (10–12). Similarly, the localization of adenosine (13), adenosine deaminase, the enzyme converting adenosine to inosine (14), adenosine-uptake sites (15), and adenosine receptors (16) to discrete regions in the brain supports a neurotransmitter-like or neuromodulatory role for adenosine in the nervous system (9, 11). It has also been reported that adenosine stimulates glycogenolysis in mouse cerebral cortical slices, increases the activity of tyrosine hydroxylase in PC12 cells, and potentiates nerve growth factor (NGF)-induced neurite outgrowth in these cells (17–19). All these effects could be mediated either by cell surface receptors (20) and/or through uptake and subsequent action of adenosine or one of its metabolites (inosine, for instance) on intracellular target sites (19, 21–23). Purines might thus have multiple and complex effects on the nervous system.

In the present report we show that the purine metabolite inosine is the main neurotrophic component present in LCM that selectively enhances the adrenergic properties of dissociated chicken sympathetic neurons in culture. Inosine acts via an intracellular pathway, as its effects can be blocked by dipyridamole, an inhibitor of nucleoside transport (24, 25). This is evidence that inosine, or one of its metabolites, may act as an intracellular neuromodulator in the nervous system.

MATERIALS AND METHODS

Cell Cultures. Superior cervical ganglia (SCG) from 9-day-old chicken embryos were dissociated and cultured as described (5). Briefly, nonneuronal cells were removed by centrifugation on a Percoll density gradient; the neurons were plated at a density of 100,000–120,000 cells per well in either Eagle's minimal essential medium containing 7S NGF at 1 μg/ml or in the same medium supplemented with LCM (6), purified LCM fractions, inosine, or adenosine (Sigma). After 10–15 days in culture, the incorporation of [^{3}H]leucine into neuronal protein (6), and the synthesis and accumulation of catecholamine and acetylcholine (AcCho) was measured after incubation with the radiolabeled precursors [^{3}H]tyrosine and [^{3}H]choline (Amersham) as described by Mains and Patterson (26).

Purification of the Neurotrophic Activity Present in LCM. *Bio-Gel P-2 chromatography.* LCM and L500, its low-molecular mass fraction, were prepared as described (5, 6). Briefly, L500 was obtained after filtering LCM through an Amicon YC05 ultrafiltration membrane having a molecular mass cutoff of 500 Da (6). Ten milliliters of the filtrate was lyophilized, resuspended in 1 ml of phosphate-buffered saline (PBS), and chromatographed on a 1 × 25 cm Bio-Gel P-2 column (Bio-Rad) equilibrated in PBS. Individual fractions (1 ml) were collected, filtered through a Millipore membrane, and added to separate neuronal culture wells at a final concentration of 5–10% (vol/vol). Neurotrophic activity (i.e., catecholamine-production-stimulating activity) was assayed by measuring neurotransmitter synthesis after 10–15 days in culture in the presence or the absence of the different fractions (not quantitated). Glutathione (307 Da), sodium metabisulfite (190 Da), and sodium chloride (58 Da) were used as molecular mass markers.

HPLC chromatography. Fraction 39 from the Bio-Gel P-2 column was further analyzed by reversed-phase HPLC using

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: SCG, superior cervical ganglion; LCM, liver-conditioned medium; L500, low-molecular-mass fraction of LCM; NGF, nerve growth factor; AcCho, acetylcholine; R_T, retention time.

†To whom reprint requests should be addressed.

a Spherisorb ODS 2 column (4.6 × 25.0 mm). A linear step gradient of 0.05% trifluoroacetic acid in acetonitrile/H_2O (9:1) with 0.05% trifluoroacetic acid in H_2O was run at a flow rate of 0.5 ml/min. The HPLC system consisted of Hewlett–Packard 1090 chromatographic equipment combined with a diode-array spectrometer that allows the simultaneous detection at 210 nm and the acquisition of a full absorption spectrum from 190 to 600 nm.

RESULTS

Purification of the low-molecular-mass fraction of LCM (L500) by chromatography on a Bio-Gel P-2 column shows that the neurotrophic activity present in L500 elutes in fractions 37–44—i.e., behind NaCl (Fig. 1). Fraction 39 was further analyzed on a Spherisorb ODS 2 HPLC column using 5'-AMP, cAMP, adenosine, inosine, hypoxanthine, and guanosine as standards. The chromatogram in Fig. 2*A* shows that fraction 39 is resolved into one major peak [retention time (R_T) = 22.2] and one minor peak (R_T = 27.7). These two peaks give different and characteristic absorption spectra (Fig. 2*A Inset*). Peak 22.2 has an absorption maximum at 248 nm. By comparing the R_T values and the absorption spectra of these two components with those of different reference compounds such as 5'-AMP (R_T = 15.4), cAMP (R_T = 21.6), adenosine (R_T = 25.4), inosine (R_T = 22.2), hypoxanthine (R_T = 16.6), and guanosine (R_T = 23.8), peak 22.2 could be identified as that of inosine. Fig. 2*B* shows the R_T values and absorption spectra of the reference compounds inosine (248 nm) and adenosine (260 nm; 1 nmol). Note that no peak corresponding to adenosine is present in L500. Peak 27.7 does not coelute with, or have the same absorption spectrum as, any of the nucleotides and nucleosides used as standards.

To test whether peak 22.2 contains all the neurotrophic activities initially present in LCM, 50 ml of L500 were lyophilized, resuspended in 1.4 ml of PBS, and chromatographed on the Bio-Gel P-2 column as described for Fig. 1. Fractions 38–42 were pooled, lyophilized, and fractionated by reversed-phase HPLC, as described for Fig. 2, by use of a preparative Spherisorb ODS 2 column (9.0 × 25.0 mm). Peaks 22.2 and 27.7 were lyophilized, resuspended in 1 ml of H_2O, and tested for the presence of neurotrophic activity. The effect of the different fractions purified from LCM and of inosine on incorporation of labeled leucine and catecholamine production in cultured chicken SCG neurons (5, 6) is summarized in Table 1. Taken together, our results show that the neurotrophic activity present in LCM is identical with inosine, a purine metabolite. Indeed, it comigrates with inosine on HPLC, has the same absorption spectrum as inosine, and stimulates incorporation of labeled leucine and catecholamine production to the same extent as inosine. It is therefore likely that the major part of the neurotrophic activity present in LCM is due to inosine.

Dipyridamole, a nucleoside-uptake blocker (24, 25), substantially decreases the stimulation of catecholamine production by L500, peak 22.2, and inosine (Table 2). Concentrations >5 × 10^{-6} M dipyridamole are toxic. Adenosine stimulates incorporation of [^{3}H]leucine into neuronal protein (A.D.Z, unpublished observation) and catecholamine production by the SCG neurons to the same extent as does inosine (Table 2). Similarly, its effects are prevented by dipyridamole, but not by theophylline, an adenosine-receptor blocker (9, 27) (Table 2).

The concentration-dependent effect of inosine on transmitter production is shown in Fig. 3. Optimal stimulations of catecholamine production are obtained at concentrations of 5 × 10^{-6} to 10^{-5} M inosine, with a half-maximal stimulation at ≈10^{-6} M. Note that catecholamine production is increased more than incorporation of [^{3}H]leucine into neuronal protein (Table 1). AcCho production, however, is increased only 181 ± 9% (n = 3)—i.e., not more than incorporation of labeled leucine (data not shown).

DISCUSSION

Purification of the neurotrophic activity present in L500 by chromatography on a Bio-Gel P-2 column allows removal of most salts present in the LCM (Fig. 1). Subsequent fractionation of the active component(s) by HPLC chromatography reveals one major peak (peak 22.2, Fig. 2), which coelutes with, and has the same absorption spectrum as, inosine, the deaminated metabolite of adenosine. Furthermore, this frac-

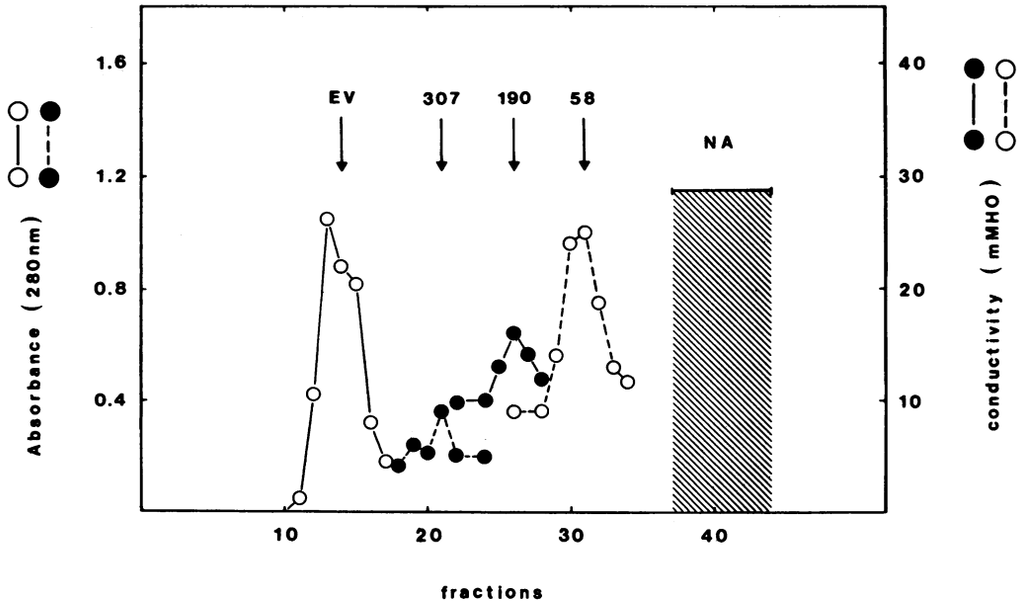

FIG. 1. Fractionation of L500 by filtration through a 1 × 25 cm Bio-Gel P-2 column equilibrated in PBS. Fractions (1 ml) were collected and tested for effects on catecholamine and AcCho production as described. The shaded area indicates fractions containing neurotrophic activity (NA, not quantitated). The arrows from left to right indicate the elution positions for blue dextran (○—○; EV, elution volume), glutathione (●---●, 307 Da), sodium metabisulfite (●—●, 190 Da), and sodium chloride (○---○, 58 Da).

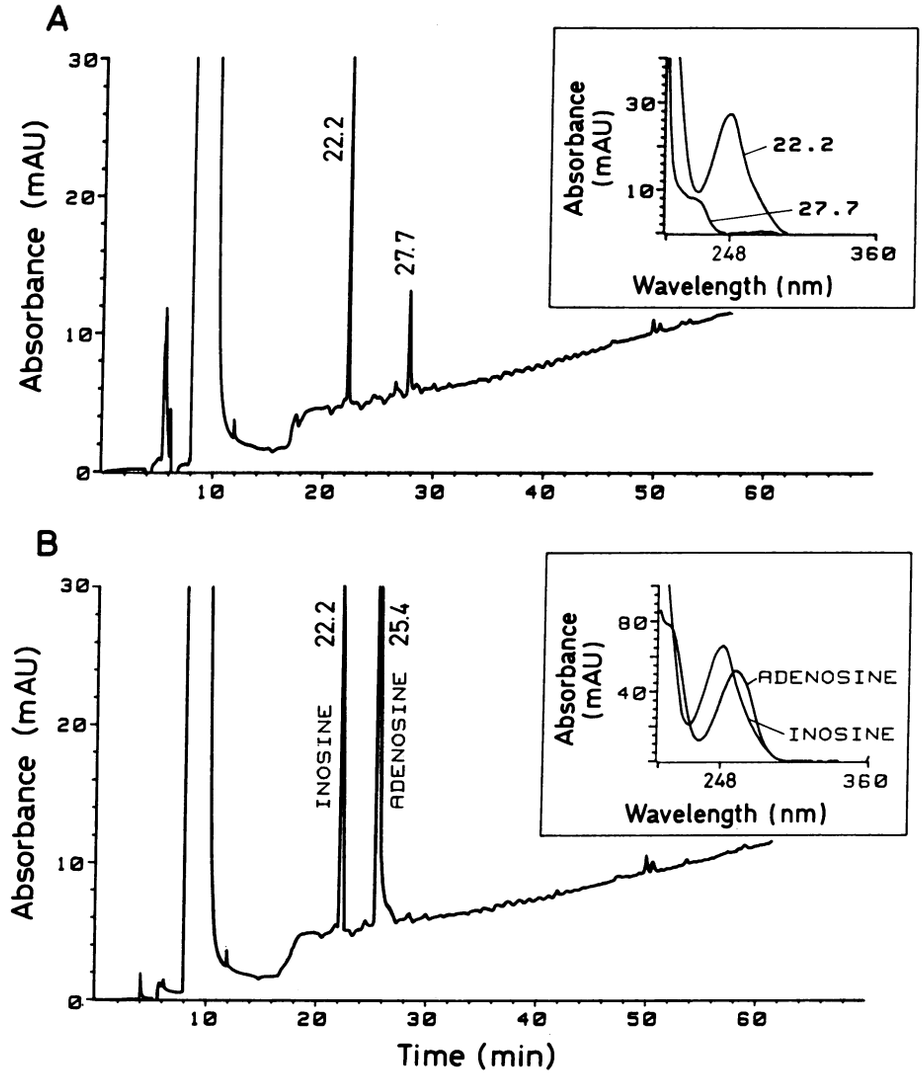

FIG. 2. HPLC chromatography on a reversed-phase Spherisorb ODS 2 column. (*A*) Analysis of fraction 39 from the Bio-Gel P-2 column. (*B*) Fractionation of inosine and adenosine (1 nmol). (*Insets*) Absorption spectra of the various compounds. mAU, milliabsorbance units.

tion, like inosine, appears to contain all the neurotrophic activities (increase in incorporation of labeled leucine and stimulation of catecholamine, but not AcCho, production) initially present in LCM (Table 1 and Fig. 3). It is therefore likely that the major part of the neurotrophic activity present in LCM is due to inosine. The fact that inosine was initially discovered in LCM is not surprising because the liver is quantitatively the major source of purines. Nucleosides or nucleotide bases released by the liver can be transported by the blood and subsequently be taken up and utilized by other tissues (28). Evidence that liver cells might induce adrenergic properties in cultured neurons has been advanced previously by Bird and James (29). These authors have described that chicken spinal cord cells cultured upon liver, but not kidney, feeder layers show intense formaldehyde-induced fluorescence and large numbers of dense-core vesicles.

Purines have been postulated to have a neurotransmitter or neuromodulatory role in the nervous system (8, 9, 30). This hypothesis is supported by the fact that (*i*) adenosine has a potent depressive effect on synaptic transmission (10–12); (*ii*) adenosine receptors, adenosine-uptake sites, and adenosine deaminase, the enzyme converting adenosine to inosine, are localized to discrete brain regions (14–16); (*iii*) nucleosides and nucleotides can be released by cultured neuronal cells upon depolarization (31, 32); (*iv*) inosine and hypoxanthine competitively inhibit binding of diazepam to brain membranes and have therefore been suggested to be endogenous ligands for the brain benzodiazepine binding sites (33). However, the mode of action of purines at the biochemical level is still not fully understood.

We present here evidence suggesting that the purine metabolite inosine can increase adrenergic development in cultured SCG neurons by acting via an intracellular pathway. Indeed, the stimulation of catecholamine production by inosine is substantially decreased by dipyridamole, a nucleoside-uptake blocker (24) (Table 2). [Note that 2×10^{-6} M dipyridamole decreases the transport of [^{3}H]adenosine by cultured neurons from chicken embryo brain by 82% (25)]. Furthermore, the stable analog of inosine, 8-bromoinosine, has no effect on the SCG neurons (A.D.Z, unpublished observation). Adenosine has the same effect as inosine on the incorporation of labeled leucine into neuronal protein and on catecholamine production. The effects of adenosine are blocked by dipyridamole, but not by theophylline, an adenosine-receptor antagonist that completely blocks the binding of adenosine to chicken brain membranes (27). This fact suggests that adenosine and/or inosine (some adenosine may be deaminated in the culture medium) are taken up by the neurons, that adenosine is converted to inosine by adenosine deaminase, and that the effect of inosine is mediated via an

Table 1. Effect of the different fractions purified from LCM and of inosine on incorporation of labeled leucine and catecholamine production

	[^{3}H]leucine per neuron, % control	Catecholamine per neuron, % control
LCM (2%)	307 ± 25	630 ± 7
L500 (2%)	279 ± 16	458 ± 53
Fractions 38–42 (Bio-Gel P-2)	246 ± 18	589 ± 21
Peak 22.2 (10^{-5} M) (HPLC)	275 ± 14	595 ± 87
Peak 27.7 (HPLC)	128 ± 7	111 ± 11
Inosine (10^{-5} M)	268 ± 24	639 ± 26

Each value represents the mean of three to six separate experiments ± SEM. All measurements were made using SCG neurons grown in parallel wells for 10–15 days in the presence or the absence (control) of the indicated components. Values are expressed as percentages of control cultures grown in the presence of NGF alone (100%); the control values ranged between 1.2–2.8 fmol per neuron per hr for [^{3}H]leucine incorporation and between 0.5–2 fmol per neuron for catecholamine production. Calculation of the concentration of peak 22.2 was based on the comparison of its absorption intensity with that of inosine. Fractions 38–42 and peak 27.7 were added at concentrations corresponding approximately to the amount in the starting material.

as-yet-unknown intracellular mechanism. Collis and colleagues (21, 22) have reported recently that adenosine-induced relaxation of the guinea pig aorta might also be mediated by inosine.

Inosine is not just a common nutrient that promotes overall protein synthesis and/or turnover in the SCG neurons, because it stimulates catecholamine production more than incorporation of labeled leucine into neuronal protein and does not specifically increase AcCho synthesis (Fig. 3) (6). [It is possible that we can see this effect because our culture medium contains only 3% serum (5), and thus this medium might be deficient in adenosine and/or inosine that is normally present in the serum.] Such a differential regulation of transmitter phenotypic expression has also been described in the presence of medium conditioned by nonneuronal cells (4), membrane depolarization (34), and cell density (5). Note that LCM does not increase incorporation of labeled leucine or

Table 2. Effect of dipyridamole and theophylline on the stimulation of catecholamine production by L500, peak 22.2, inosine, and adenosine

	Catecholamine production per neuron, % control		
	Control	Dipyridamole (5×10^{-6} M)	Theophylline (10^{-5} M)
L500 (1%)	503 ± 70 (n = 8)	288 ± 62 (n = 4)	645 ± 178 (n = 4)
Peak 22.2 (10^{-5} M)	541 ± 43 (n = 6)	170 ± 20 (n = 3)	ND
Inosine (10^{-6} M)	396 ± 42 (n = 9)	197 ± 13 (n = 4)	585 ± 37 (n = 5)
Inosine (10^{-5} M)	664 ± 89 (n = 7)	190 ± 2 (n = 2)	668 ± 59 (n = 3)
Adenosine (10^{-6} M)	419 ± 77 (n = 8)	166 ± 48 (n = 3)	503 ± 28 (n = 3)
Adenosine (10^{-5} M)	596 ± 61 (n = 11)	229 ± 91 (n = 3)	506 ± 28 (n = 4)

All measurements were made using SCG neurons grown in parallel wells for 10–15 days in the presence or the absence (control) of the indicated components. Values are expressed in percentages of control cultures (0.5–2 fmol per neuron) grown in the presence of NGF alone. Each value represents the mean of n separate experiments ± SEM. ND, not determined.

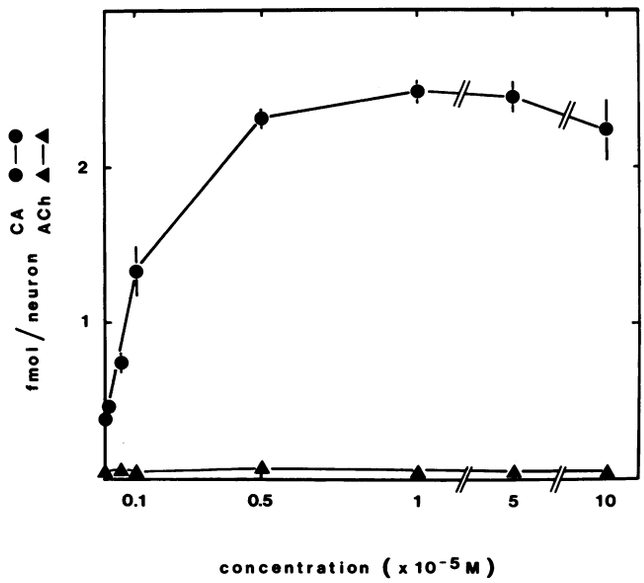

FIG. 3. Catecholamine (CA, ●) and AcCho (ACh, ▲) production in SCG neurons grown for 2 weeks in the presence of increasing concentrations of inosine. Transmitter synthesis and accumulation were measured after a 4-hr incubation in the presence of [^{3}H]tyrosine and [^{3}H]choline and expressed in fmol per neuron. Each point represents the mean of two separate experiments, and the vertical bars indicate SEM.

induce catecholamine production in cultured chicken ciliary ganglion, dorsal root ganglion, or spinal cord neurons (5).

We have previously shown that high NGF concentrations potentiate the stimulating effect of LCM—i.e., inosine, on catecholamine production (6). Similarly, it has been reported that NGF-induced neurite outgrowth is potentiated by another purine compound, cAMP (35). Interestingly, this effect appears to be mediated by the cAMP metabolites adenosine and inosine (19). Because they have a synergistic effect on neurite outgrowth and catecholamine production, inosine and NGF seem to act through different mechanisms.

The biochemical pathways involved in the action of inosine on the SCG neurons need further investigation. Inosine might be converted to hypoxanthine, IMP, AMP, and finally ATP (purine salvage pathway). This is indeed the case in cultured rat sympathetic neurons, where 70–80% of the label is associated with ATP after uptake of [^{3}H]adenosine or [^{3}H]-adenine (31, 36). Intracellular ATP itself, rather than its hydrolysis products, has recently been discovered to directly control the activity of ion channels—i.e., membrane glycoproteins that are involved in many cellular functions (37, 38). Alternatively, ATP might modulate the activity of protein kinases, as demonstrated for Ca^{2+}/calmodulin-dependent protein kinase isolated from rat brain (39). It is also possible that a metabolite of inosine can regulate the levels of mRNA encoding neurotransmitter-synthesizing enzymes (1).

In summary, we present here evidence that suggests inosine or one of its metabolites, might be an environmental signal capable of regulating neurotransmitter phenotypic expression. The identification of inosine as a modulator of adrenergic properties in cultured SCG neurons has brought us one step closer to understanding some of the intracellular mechanisms that might mediate the regulation of catecholaminergic traits by environmental stimuli.

We thank Drs. M. Cuenod and A. Kato for helpful comments on the manuscript. This work was supported by the Swiss National Foundation for Scientific Research (grants 3.151-0.85 and 3.389-1.86) and the Dr. Eric Slack-Gyr Foundation.

1. Black, I. B., Adler, J. E., Dreyfus, C. F., Friedman, W. F., LaGamma, E. F. & Roach, A. H. (1987) *Science* **236,** 1263–1268.
2. Xue, Z. G., Smith, J. & Le Douarin, N. M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8800–8804.
3. Howard, M. J. & Bronner-Fraser, M. (1986) *Dev. Biol.* **117,** 45–54.
4. Patterson, P. H. & Chun, L. L. (1977) *Dev. Biol.* **56,** 263–280.
5. Zurn, A. D. & Mudry, F. (1986) *Dev. Biol.* **117,** 365–379.
6. Zurn, A. D. (1987) *J. Neurosci.* **7,** 3566–3573.
7. Fukada, K. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8795–8799.
8. Su, C. (1983) *Annu. Rev. Pharmacol. Toxicol.* **23,** 397–411.
9. Snyder, S. H. (1985) *Annu. Rev. Neurosci.* **8,** 103–124.
10. Silinsky, E. M. (1984) *J. Physiol. (London)* **346,** 243–256.
11. Dunwiddie, T. V. (1985) *Int. Rev. Neurobiol.* **27,** 63–140.
12. Prestwich, S. A., Forda, S. R. & Dolphin, A. C. (1987) *Brain Res.* **405,** 130–139.
13. Braas, K. M., Newby, A. C. Wilson, V. S. & Snyder, S. H. (1986) *J. Neurosci.* **6,** 1952–1961.
14. Geiger, J. D. & Nagy, J. I. (1986) *J. Neurosci.* **6,** 2707–2714.
15. Bisserbe, J. C., Patel, J. & Marangos, P. J. (1985) *J. Neurosci.* **5,** 544–550.
16. Goodman, R. R. & Snyder, S. H. (1982) *J. Neurosci.* **2,** 1230–1241.
17. Magistretti, P. J., Hof, P. R. & Martin, J. L. (1986) *J. Neurosci.* **6,** 2558–2562.
18. Erny, R. E., Berezo, M. W. & Perlman, R. L. (1981) *J. Biol. Chem.* **256,** 1335–1339.
19. Braumann, T., Jastorff, B. & Richter-Landsberg, C. (1986) *J. Neurochem.* **47,** 912–919.
20. Stiles, G. L. (1986) *Trends Pharm. Sci.* **7,** 486–490.
21. Collis, M. G. & Brown, C. M. (1983) *Eur. J. Pharmacol.* **96,** 61–66.
22. Collis, M. G., Palmer, D. B. & Baxter, G. S. (1986) *Eur. J. Pharmacol.* **121,** 141–145.
23. Lohse, M. J., Maurer, K., Gensheimer, H. P. & Schwabe, U. (1987) *Naunyn-Schmiedebergs Arch. Pharmakol.* **335,** 555–560.
24. Roos, H. & Pfleger, K. (1972) *Mol. Pharmacol.* **8,** 417–425.
25. Thampy, K. G. & Barnes, E. M. (1983) *J. Neurochem.* **40,** 874–879.
26. Mains, R. E. & Patterson, P. H. (1973) *J. Cell Biol.* **59,** 329–345.
27. Barnes, E. M. & Thampy, K. G. (1982) *J. Neurochem.* **39,** 647–652.
28. Pritschard, J. B. (1975) *Am. J. Physiol.* **229,** 967–973.
29. Bird, M. M. & James, D. W. (1975) *J. Neurocytol.* **4,** 633–646.
30. Burnstock, G. & Brown, C. M. (1981) in *Receptors and Recognition*, ed. Burnstock, G. (Chapman and Hall, New York), Series B, Vol. 12, pp. 1–45.
31. Wolinsky, E. J. & Patterson, P. H. (1985) *J. Neurosci.* **5,** 1680–1687.
32. Furshpan, E. J., Potter, D. D. & Matsumoto, S. G. (1986) *J. Neurosci.* **6,** 1099–1107.
33. Asano, T. & Spector, S. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 977–981.
34. Kessler, J. A., Adler, J. E., Bohn, M. C. & Black, I. B. (1981) *Science* **214,** 335–336.
35. Heidemann, S. R., Joshi, H. C., Schechter, A., Fletcher, J. R. & Bothwell, M. (1985) *J. Cell Biol.* **100,** 916–927.
36. Tolkovsky, A. M. & Suidan, H. S. (1987) *Neuroscience* **23,** 1133–1142.
37. Spruce, A. E., Standen, N. B. & Stanfield, P. R. (1985) *Nature (London)* **316,** 736–738.
38. Ashcroft, F. M. (1988) *Annu. Rev. Neurosci.* **11,** 97–118.
39. Lou, L. L., Lloyd, S. J. & Schulman, H. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9497–9501.