Fukushima, C. A. Nichol, E. Diliberto, Jr., O. H. Viveros, *Soc. Neurosci. Abstr.* **6**, 643 (1980); O. H. Viveros, C.-L. Lee, M. M. Abou-Donia, J. C. Nixon, C. A. Nichol, *Science* **213**, 349 (1981).
15. W. P. Bullard, P. B. Guthrie, P. V. Russo, A. J. Mandell, *J. Pharmacol. Exp. Ther.* **206**, 4 (1978).
16. G. Kapatos and S. Kaufman, *Science* **212**, 955 (1981).
17. R. A. Levine, D. M. Kuhn, A. C. Williams, W. Lovenberg, in *The Influence of Age on the Pharmacology of Psychoactive Drugs*, A. Raskin, D. S. Robinson, J. Levine, Eds. (Elsevier/North-Holland, New York, in press).
18. W. Lovenberg, R. A. Levine, L. P. Miller, in *Second Conference on Monoamine Enzymes*, E. Usdin, N. Weiner, M. Youdim, Eds. (Macmillan, New York, in press).
19. U. Ungerstedt, *Eur. J. Pharmacol.* **5**, 107 (1968).
20. S. Pradhan, L. Alphs, W. Lovenberg, *Neuropharmacology* **20**, 149 (1981).
21. Supported by a grant to L.P.M. from the Dystonia Medical Research Foundation. R. A. L. is a predoctoral student in the Department of Pharmacology, George Washington University Medical Center, Washington, D.C.

17 April 1981; revised 14 July 1981

Renin and Angiotensin: The Complete System Within the Neuroblastoma × Glioma Cell

Abstract. *Cells of the homogeneous hybrid line neuroblastoma × glioma (NG108-15) have many neuronal properties. Immunocytochemical tests show that they contain both immunoreactive renin and angiotensin; direct radioimmunoassays show that they are positive for renin, angiotensin I, and angiotensin II; enzymatic assays show that they contain angiotensinogen and converting enzyme as well. The renin appears to be present in an enzymatically inactive form that can be activated by trypsin and then blocked by antiserum to purified mouse submaxillary renin. Renin concentration and activity are increased by enhancing cellular differentiation with dibutyryl cyclic adenosine monophosphate or by serum withdrawal. These findings demonstrate a complete renin-angiotensin system within these neuron-like cells, and suggest that activation of intracellular renin could generate angiotensin II.*

The peripheral renin-angiotensin system is integral to blood pressure control and extracellular fluid volume homeostasis (*1*). The active hormone angiotensin II is generated within the circulation by sequential cleavage by renal renin of liver-derived angiotensinogen to form angiotensin I, and subsequent conversion of angiotensin I to angiotensin II by converting enzyme. Several lines of evidence suggest the possibility of a separate renin-angiotensin system intrinsic to the central nervous system (*2–5*). Exogenous administration of renin, angiotensin I, or angiotensin II into the brain ventricles raises blood pressure, stimulates drinking, and releases antidiuretic hormone and adrenocorticotropin (*2, 6*). Renin enzymatic activity, and renin and angiotensin immunoreactivity have been demonstrated in several regions of the brain (*4, 5*). It is not known whether neural tissue generates renin and angiotensin, or rather utilizes the components synthesized within kidney and liver. Also unknown is whether the renin-angiotensin cascade is completed within the circulation of the brain and then the products absorbed by neural tissue or whether some of the steps in angiotensin II generation can be completed within the nerve cells.

We have investigated a homogeneous cell line in tissue culture, the neuroblastoma × glioma (NG108-15) cell, as a possible model for understanding the renin-angiotensin system in neurons. Because of their nervelike properties these cells have proved helpful in investigations of neurotransmitter synthesis, synapse formation, and membrane excitability (*7*). We report here that they contain antigenic renin that must be activated in order to allow angiotensin I to be generated. They also contain a renin substrate, converting enzyme, angiotensin I, and angiotensin II, suggesting that the complete renin-angiotensin cascade may be completed within a single cell.

Cells of the NG108-15 line of mouse neuroblastoma × rat glioma were grown and passaged in Dulbecco's modification of Eagle's minimum essential medium, supplemented with 5 percent fetal bovine serum, $1 \times 10^{-4}M$ hypoxanthine, $1 \times 10^{-6}M$ aminopterin, and $1.6 \times 10^{-5}M$ thymidine. For radioimmunoassay, cells were washed three times with phosphate-buffered saline, sonicated for 9 seconds on ice, and immediately frozen in phosphate-buffered saline in a bath containing acetone and dry ice. The radioimmunoassay and immunocytochemistry for renin were performed with rabbit antibody to pure renin from mouse submaxillary gland. This renin was purified by the method of Cohen *et al.* (*8*) and appeared as a single band on both sodium dodecyl sulfate (SDS) and polyacrylamide gels. Its biochemical similarity to renal renin has been established (*9*). The direct renin radioimmunoassay was developed according to the method of Michelakis *et al.* (*10*) with iodination by the chloramine-T method. Sensitivity was 50 pg. Angiotensin I and angiotensin II radioimmunoassays were performed by the methods of Poulsen and Jorgensen (*11*) and Nussberger *et al.* (*12*), respectively.

Table 1. All components of the renin-angiotensin system are located in the NG108-15 cell. Only trace amounts of angiotensin I were generated before the cells were exposed to trypsin; the levels reported are those obtained after the cells were trypsinized by the method of Cooper *et al.* (*21*). Trypsinization was terminated after 20 minutes by soybean trypsin inhibitor. Control cells exposed to trypsin that had previously been mixed with inhibitor showed no activity. The values shown here were obtained without the addition of exogenous substrate; values were comparable with its addition. Antibody inhibition experiments were performed with the antiserum diluted 1:1000. The radioassay for converting enzyme was performed with [^{3}H]Hip-Gly-Gly used as substrate and ethyl acetate used for extraction at *p*H 8.0. In each case the converting enzyme inhibitor SQ 14,225 ($10^{-3}M$) blocked the converting enzyme activity of the cells. Cells were exposed to dibutyryl cyclic AMP (1 m*M*) in serum-containing medium for 4 days. Each assay was performed in duplicate and is reported as ± 1 standard deviation; *N* is the number of independent assays. The symbol (+) indicates that angiotensin I could be generated without exogenous substrate.

Group	Renin			Substrate (N = 4)	Angiotensin II (N = 2) (fmole/mg protein)	Converting enzyme	
	Concentration (N = 2) (pg/mg protein)	Activity* (N = 4)	Inhibition by antibody (%)			Activity (N = 3) (U/mg)	Inhibition by 10^{-3} M SQ 14,225 (%)
Control	34 ± 10	200 ± 30	87	+	62 ± 5	5.5 ± 0.1	91
Serum-free	132 ± 25	1000 ± 30	90	+	32 ± 3	5.5 ± 0.1	88
Dibutyryl cyclic AMP	Not tested	300 ± 20	92	+	99 ± 2	5.8 ± 0.1	96

*Measured as picograms of angiotensin I per milligram of protein per hour.

0036-8075/81/1120-0921$01.00/0

The radioimmunoassay for angiotensin I was performed after the cells were incubated for 3 hours at 37°C, *p*H 7.4, in the presence of EDTA (Mallinckrodt), *N*-ethylmaleimide (Sigma), 8-hydroxyquinoline (Eastman), diisopropylfluorophosphate (Sigma), dimercaptol (Sigma), and SQ 14,225 (Squibb), each at $10^{-3}M$. The sensitivity was 5 pg. The radioimmunoassay for angiotensin II included EDTA (2.6 m*M*), phenylmethylsulfonylfluoride (1 m*M*), tris (0.2*M*), and bovine serum albumin (0.2 percent), and was performed at *p*H 7.5; sensitivity was 5 fmole. Rabbit antiserum to [Val^5]angiotensin II, which cross-reacts with [Ile^5]angiotensin II, was used for immunocytochemistry (*13*). Immunoreactive renin and angiotensin II were detected by the indirect peroxidase-antiperoxidase method (*14*) after the cells were washed in buffer and fixed with 3.6 percent Formalin for 40 minutes.

The distribution of renin and angiotensin within the NG108-15 cells, as shown by immunocytochemistry, is illustrated in Fig. 1, along with control plates on which the antibody had been preabsorbed with purified mouse submaxillary gland renin or synthetic angiotensin II (Beckman), respectively. Nearly every cell contains immunoreactive renin and immunoreactive angiotensin; there is no evidence for restriction of the distribution of either to soma or processes.

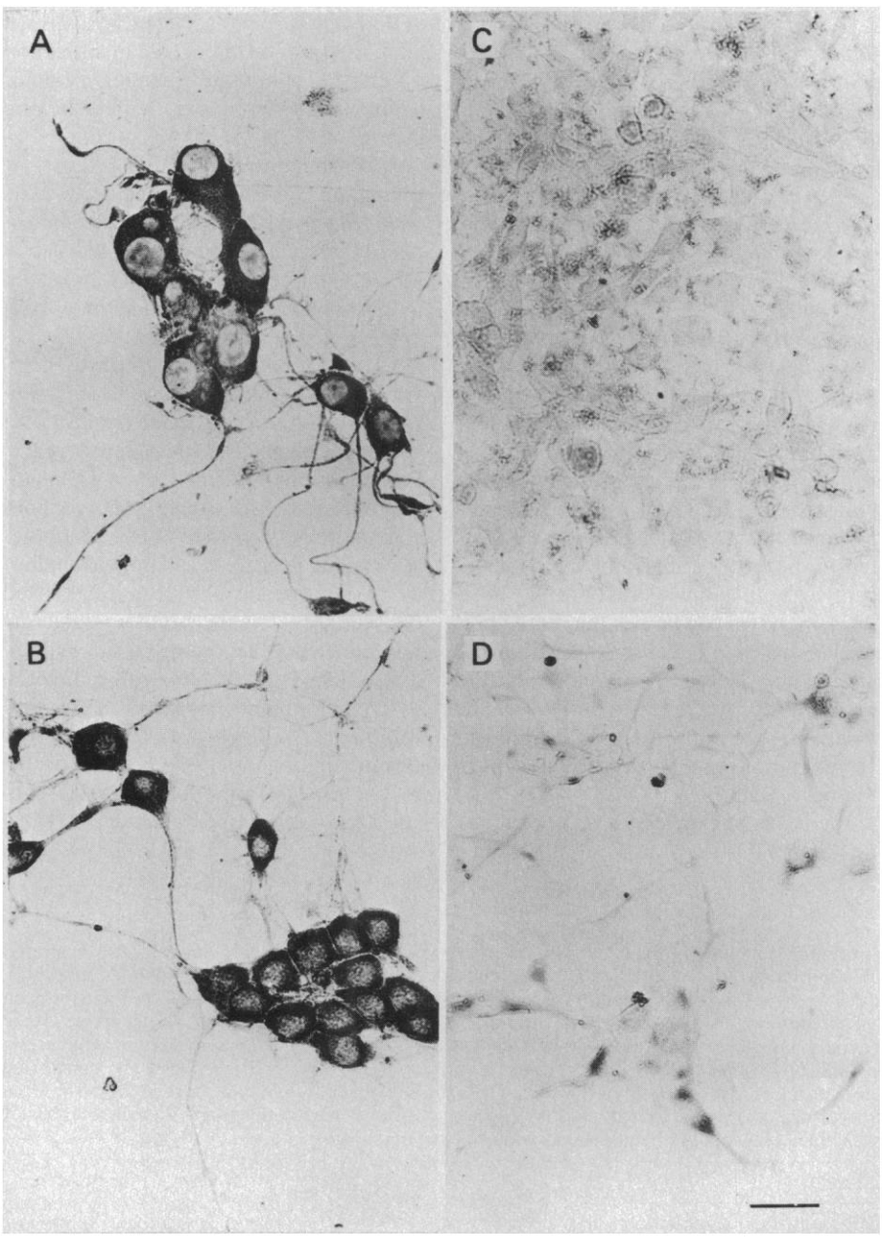

Fig. 1. (A) Immunoreactive renin and (B) immunoreactive angiotensin II in sister cultures of NG108-15 cells. The immunoperoxidase technique was performed by incubating the cells with primary antiserum at 4°C for 12 hours. Reaction products are distributed throughout the cytoplasm, within cell bodies and processes. (C and D) Controls for specificity showing absence of staining when antibodies are preabsorbed with the respective antigens: (C) 1 ml containing antibody (1:400 by volume), and renin previously incubated with 100 μg of purified renin, and (D) 1 ml containing antibody (1:800 by volume), and angiotensin II previously incubated with 40 μg of synthetic [Ile^5]angiotensin II. (Scale bar, 50 μm.)

To confirm this observation, we measured renin, angiotensin I, and angiotensin II by radioimmunoassay. The results are shown in Table 1. The amount of renin the NG108-15 cells contain (approximately 34 pg per milligram of protein) is comparable to that reported in several areas of the brain (*2, 4*). Only trace amounts of angiotensin I are identified in the sonicated cells when they are incubated at 37°C either with or without the addition of exogenous substrate. By contrast, after trypsinization the cells contain approximately 200 pg of angiotensin I per milligram of protein per hour. This generation of angiotensin I is completely inhibited by antiserum to pure renin from mouse submaxillary gland. The cells contain approximately 60 fmole of angiotensin II per milligram of protein. No renin activity is found in control baby hamster kidney (BHK) cells, with or without exogenous substrate before or after trypsinization, nor is angiotensin II detected in them.

To address the possibility that the renin and angiotensin II might have been derived from the fetal calf serum in the medium, cells were grown in a defined serum-free medium (*15*) for 1 week. These cells still contain antigenic renin, trypsin-activated renin activity, angiotensin II, and converting enzyme (Table 1). Renin concentrations are increased over control values, as measured both by direct radioimmunoassay and by the assay for enzymatic activity. Serum withdrawal has been shown to enhance morphological and electrophysiological differentiation in neuroblastoma cells (*16*). Dibutyryl cyclic adenosine monophosphate also induces this type of differentiation (*17*), and also causes an increase in renin activity without inducing any obvious changes in the cellular distribution of renin as revealed by immunocytochemistry.

Other components that would be needed to generate angiotensin II within the cell are renin substrate and converting enzyme. Since angiotensin I is generated without the addition of exogenous substrate, it is apparent that the cells contain an angiotensinogen. Converting enzyme was measured by radioassay with [^{3}H]Hip-Gly-Gly being used as the sub-

strate (Ventrex Laboratories) and the presence of the enzyme confirmed by inhibition with the converting enzyme inhibitor SQ 14,225 (Table 1).

Thus these cells contain all of the components of the complete renin-angiotensin system. Renin appears to be present in an inactive form. Although some investigators equate trypsin-activated renin with prorenin (*4*), the possibility that it represents renin bound to a protein inhibitor (*18*) cannot be excluded entirely. Inactive renin in plasma can be activated in vitro by exposure to an acid *p*H or to a variety of proteolytic enzymes including trypsin, kallikrein, and nerve growth factor (*19*). However, it is not established that this activation occurs in vivo. Similarly, the nature of this inactive renin and its endogenous mechanism of activation remains unknown. NG108-15 cells, a line of relatively homogeneous cells that express many properties of differentiated neurons, may prove useful in studies of the control of synthesis, secretion, and mechanism of action of renin and angiotensin within the nervous system.

The widespread presence of all components of the renin-angiotensin system in brain suggests that the role of this system may encompass more than blood pressure and volume regulation. The potential linkage of renin and angiotensin to processes of neuronal excitation (*20*) remains unexplored. It is possible that the entire renin-angiotensin cascade can be completed within a single nerve cell, and that control of the activation of intracellular renin might be a regulatory step in this pathway.

MARK C. FISHMAN*
Laboratory of Developmental Biology, National Institutes of Health, Bethesda, Maryland 20205
EARL A. ZIMMERMAN
Department of Neurology, College of Physicians and Surgeons, Columbia University, New York 10032
EVE E. SLATER
Medical Services, Massachusetts General Hospital, Boston, Massachusetts 02114

References and Notes

1. I. H. Page and J. W. McCubbin, *Renal Hypertension* (Yearbook, Chicago, 1969); R. Tigerstedt and P. G. Bergman, *Skand. Arch. Physiol.* **8**, 223 (1898).
2. M. I. Phillips, J. Weyhenmeyer, D. Felix, D. Ganten, W. Hoffman, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 2260 (1979); I. Reid, *ibid.*, p. 2225; M. I. Phillips, *Neuroendocrinology* **25**, 354 (1978); D. Ganten and G. Speck, *Biochem. Pharmacol.* **27**, 2379 (1978); T. Inagami, S. Hirose, H. Yokosawa, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 636 (Abstr.) (1979).
3. S. Hirose, H. Yokosawa, T. Inagami, *Nature (London)* **274**, 392 (1978).
4. _____, R. Workman, *Brain Res.* **191**, 489 (1980).
5. E. E. Slater, R. Defendini, E. A. Zimmerman, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5438 (1980).
6. B. Anderson and O. Westbye, *Life Sci.* **9**, 601 (1970).
7. T. Amano, E. Richelson, M. Nirenberg, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 258 (1972); P. Nelson, C. Christian, M. Nirenberg, *ibid.* **73**, 123 (1976).
8. S. Cohen, J. Taylor, K. Murakami, A. Michelakis, T. Inagami, *Biochemistry* **11**, 4286 (1972).
9. E. E. Slater, *Methods Enzymol.* **34**, 427 (1981).
10. A. Michelakis, H. Yoshida, J. Menzie, K. Murakami, T. Inagami, *Endocrinology* **94**, 1101 (1974).
11. K. Poulsen and J. Jorgensen, *J. Clin. Endocrinol. Metab.* **39**, 816 (1974).
12. J. Nussberger, R. Re, G. Matsueda, E. Haber, in preparation.
13. M. M. Kilcoyne, D. L. Hoffman, E. A. Zimmerman, *Clin. Sci.* **59**, 57S (1980).
14. L. A. Sternberger, *Immunocytochemistry* (Prentice-Hall, Englewood Cliffs, N.J., 1974).
15. Serum-free medium consisted of Ham's F12 medium supplemented with insulin (5 μg/ml), transferrin (100 μg/ml), putrescine (100 μ*M*), selenium (30 n*M*), and progesterone (20 n*M*) [J. E. Bottenstein and G. H. Sato, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 514 (1979)]. For these experiments cells were washed free of serum-containing medium twice over 30 minutes and then grown for 1 week with three changes of medium before analysis.
16. J. Minna. P. Nelson, J. Peacock, D. Glazer, M. Nirenberg, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 234 (1971); N. W. Seeds, A. G. Gilman, T. Amano, M. Nirenberg, *ibid.* **66**, 160 (1970).
17. B. Hamprecht, T. Amano, M. Nirenberg, unpublished data; A. Chalazonitis, J. Minna, M. Nirenberg, *Exp. Cell Res.* **105**, 269 (1977).
18. K. Poulsen, J. Kroll, A. H. Neilson, J. Jensenius, C. Malling, *Biochim. Biophys. Acta* **577**, 1 (1979).
19. J. Sealey, S. Atlas, J. Laragh, N. Oza, J. Ryan, *Nature (London)* **275**, 144 (1978); N. Yokosawa, N. Takahashi, T. Inagami, D. Page, *Biochim. Biophys. Acta* **569**, 211 (1976); B. Morris and D. Catanzaro, *IRCS Med. Sci. Physiol.* **8**, 433 (1980); B. J. Leckie and N. K. McGhee, *Nature (London)* **288**, 702 (1980).
20. M. C. Fishman, *Nature (London)* **260**, 542 (1976).
21. R. M. Cooper, G. E. Murray, D. H. Osmond, *Circ. Res.* **40**, I-171 (1977).
22. We thank P. G. Nelson and M. Nirenberg for helpful suggestions, M. Nirenberg for the NG108-15 cells, M. Karnovsky for the BHK cells, J. Nussberger for the angiotensin II antiserum, Squibb for the SQ 14,225, and S. Rosario, E. Piel, and H. V. Strout, Jr., for expert assistance. Supported in part by grants HL-21247 and HL-24105 from the National Institutes of Health. E.E.S. is an Established Investigator of the American Heart Association.
* Present address: Department of Medicine, Massachusetts General Hospital, 32 Fruit Street, Boston 02114.

1 May 1981; revised 12 August 1981

Both μ and δ Opiate Receptors Exist on the Same Neuron

Abstract. *Low concentrations of the relatively selective opiate receptor agonists dihydromorphine and normorphine (μ receptor agonists) and D-Ala²-D-Leu⁵-enkephalin (a δ receptor agonist) were applied to single enteric neurons while the frequency of action potential firing was recorded. Most neurons that were inhibited by the μ agonists were also inhibited by the δ agonist, but the two receptors could be distinguished by the higher concentration of naloxone required to antagonize the δ agonist. The results indicate that enteric neurons bear both μ and δ receptors and that cell firing is inhibited if either receptor type is activated.*

Plant opiates, synthetic opiate-like drugs, the naturally occurring opioid peptide enkephalin, and many enkephalin analogs all bind to a limited number of sites on neuronal membranes (*1–3*). Studies of the displacement of labeled ligands by unlabeled ligands (*3, 4*) and experiments in which binding site alkylation is prevented by prior exposure to an unlabeled ligand (*5, 6*) have provided convincing evidence that two binding sites can be distinguished. Pharmacological experiments also indicate two distinct sites, termed μ and δ receptors (*3*). The existence of two types of opiate receptors raises the question of whether they are borne by discrete neuronal populations and whether the functional con-

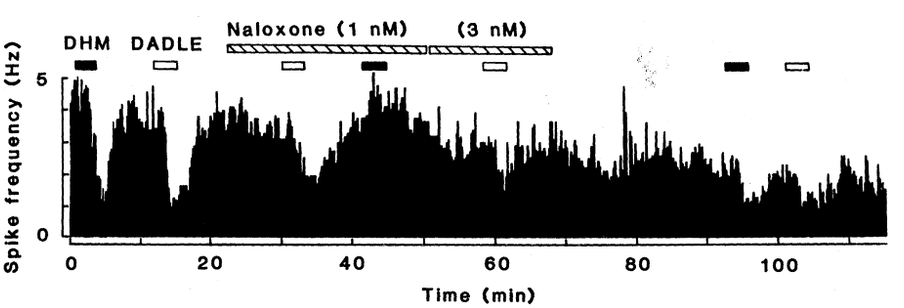

Fig. 1. Inhibition of neuronal firing by DHM (10 n*M*) and DADLE (1 n*M*). Binding studies carried out on membrane homogenates in tris buffer indicate that the ligands bind to distinct sites at these concentrations (*8*). Both agonists caused approximately equal but submaximal depression of firing. Naloxone (1 n*M*) completely prevented the effect of DHM but had little effect on the inhibition due to DADLE. A slightly higher concentration of naloxone (3 n*M*) largely prevented the effect of DADLE. Both agonists again inhibited cell firing after washout of the naloxone. The control firing rate of this neuron slowly declined during the 2-hour recording period.

0036-8075/81/1120-0923$01.00/0