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11. The topography of the 30-m^2 plots was level, not sloping more than 5 percent. Soils in the study area were from similar volcanic origin. The canopy coverage was analyzed on 18 and 19 November on three transects of 30 m in each plot. Results showed that the dominant tree in the study area is *Metrosideros collina* and that *Andropogon virginicus*, *Styphelia tamaiemaie*, and *Dodonaea eriocarpa* account for 90 percent of the understory canopy coverage in each plot. We assumed that SO_2 caused visible injury to the vegetation near Pauahi Crater, because no other noxious gases are emitted from Hawaiian volcanoes and there were no signs of lava, dustfall, or heat stress in any of the plots. Each time the canopy coverage of a species was recorded along the transects a value ranging from 0 to 3 was also recorded to score visual injury. Scoring was determined as follows: 0, plant with no apparent injury; 1, plant more alive than dead (less than half the leaf area appeared necrotic or the whole plant was slightly chlorotic); 2, plant more dead than alive (more than half the leaf area appeared necrotic or the whole plant was severely chlorotic); and 3, plant was dead.
12. We determined the SO_2 concentrations with five sample measurements, using an MSA universal sampling pump and Gastec analyzer tubes (Mine Safety Appliances Company, Pittsburgh, Pa.) on loan from S. Siegel, Department of Botany, University of Hawaii, Honolulu.
13. Stomatal conductance measurements were made with a Lambda diffusive resistance porometer (Lambda Instruments, Lincoln, Neb.).
14. Survey made by C. W. Smith, Botany Department, University of Hawaii, Honolulu.
15. We thank many scientists at the University of Hawaii (UH) in Honolulu, at Hawaii Volcanoes National Park (HVNP) and at Hawaii Volcano Observatory (HVO) for their help. We are particularly indebted to Drs. C. W. Smith and S. Siegel (UH), K. Baker (HVNP), and T. Casadevall (HVO). We also thank B. Lilley for drafting figures and R. Goldstein for his comments on an early version of this report. Research was supported by an Electrical Power Research Institute grant (RP-1313) to H.A.M.

22 July 1980

Visualization of Specific Angiotensin II Binding Sites in the Brain by Fluorescent Microscopy

Abstract. *The organum vasculosum of the lamina terminalis has been implicated as the site of receptors mediating central responses of angiotensin II. Up to now, this had been based on indirect evidence, but direct visualization of angiotensin II at its site of action has now been achieved by the use of a biologically active fluorescent angiotensin II agonist. The ventricular surface of the organum vasculosum lamina terminalis showed intense fluorescence, which was virtually eliminated by an excess of unlabeled angiotensin II.*

When angiotensin II is injected into the brain, drinking and other responses related to water balance are elicited (*1*). Evidence from both in vivo and in vitro studies implicates the organum vasculosum of the lamina terminalis (OVLT) as the site of receptors mediating these central responses to angiotensin II (*2*). When the ependymal surface of this region is physically blocked by a cream plug, the responses to intraventricularly administered angiotensin II are abolished (*3*). When angiotensin II is injected into the ventricle close to the OVLT, low concentrations are required to produce effects equal to higher concentrations injected into other parts of the brain ventricles (*4*). Lesions in the OVLT area produce a syndrome of transient adipsia and loss of responsiveness to angiotensin II injections (*5*). In addition, hypertension fails to develop in rats after lesions in this area (*5*). Binding assays carried out in vitro revealed specific binding sites for angiotensin II in the OVLT, with increased angiotensin II binding capacity in spontaneously hypertensive rats (*6*). Microiontophoretic studies in anesthetized rats showed specific activation of cells in the OVLT when angiotensin II was applied directly (*7*). These studies, however, provide only indirect evidence that the OVLT is the receptor site for angiotensin II. We now present direct evidence, obtained by a technique that combines the use of a biologically active structural analog of angiotensin II and a competitive binding control in vivo.

Fluorescein thiocarbamyl (FTC) was conjugated to angiotensin II (Meloy Laboratories) in an initial molar ratio of 5:1 in the reaction mixture, with a final maximum ratio of 1:1, by a modification of the method of Nairn (*8*); the *p*H of the reaction mixture was 8.0. The conjugate was separated by gel filtration on a Sephadex G-15 column that had been equilibrated and eluted with 0.001*M* acetic acid. The conjugate was isolated as a homogenous peak corresponding to authentic angiotensin II. Free FTC was eluted 50 ml later. The delayed elution of FTC was due to its property to adsorb on Sephadex G-15 and resulted in a clear-cut separation of the conjugate from free fluorescein.

Ultraviolet spectrum analysis was used to characterize the conjugate and to determine the ratio of FTC to protein. Fluorescent polarization analysis (performed by Meloy Laboratories) demonstrated that no free angiotensin II was present in the conjugate. In a competition assay, the binding of the conjugate to antibodies to angiotensin II was identical to binding of authentic angiotensin II.

Lateral ventricle cannulation was performed on eight adult male Sprague-Dawley rats with use of 23-gauge steel tubing stabilized by Cranioplastic. Rats had at least 3 days to recover from surgery, and patency of the cannulas was tested at least 1 hour before experimentation. Cannulas were judged to be patent if drinking behavior was elicited by a 100-ng dose of angiotensin II delivered in 1 μl of 0.9 percent saline. A 0.96 m*M* stock solution of the conjugate was prepared in 0.9 percent saline so that 1 μl contained a molar concentration tenfold higher than that of the angiotensin II solution containing 100 ng/μl.

Each of five adult male Sprague-Dawley rats was injected with 1 μl of a 1 percent solution of bovine serum albumin (BSA) (to decrease nonspecific binding) and, after a 5-minute interval, with an injection of 1.4 μg of conjugate in 1 μl of saline. Drinking behavior was elicited within 2 minutes after the injection of conjugate. The animals were decapitated during the time of drinking. The brains were removed within 3 minutes and immersed unfixed in liquid nitrogen. Cryostat sections were taken at 16 μm in the horizontal plane, and every fourth section was mounted with a buffered glycerol solution for fluorescence microscopy. Cutting began ventrally at the optic chiasm and included all of the OVLT and the ventral portion of the subfornical organ. To saturate the angiotensin II receptors with excess unlabeled angiotensin II, three rats were injected with 1 μl of 1 percent BSA and 5 minutes later with 8 μl of an angiotensin II solution (6 μg per microliter of 0.9 percent saline). Thirty seconds after injection of angiotensin II, 1 μl of the conjugate solution was injected. After drinking behavior was observed in each animal, the animals were killed and the brains were removed and processed as before.

The brains of all rats receiving the conjugate exhibited intense fluorescence that was limited to the ventricular surface of the OVLT (Fig. 1). The adjacent third ventricle showed low-level fluorescence along the ependymal wall (Fig.

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1B). Background fluorescence appeared to be due to nonspecific binding of the conjugate to the lateral walls of the third ventricle, because the conjugate was not displaced by excess angiotensin II. The brain tissue was otherwise dark. The BSA injection decreased background fluorescence, which accentuated the borders of the specific angiotensin II binding region. The borders of this region were easily discernible in the absence of BSA injection, but were not as clearly defined. Excess unlabeled angiotensin II eliminated the fluorescence from the ventricular surface of the OVLT.

Displacement of the conjugate from the OVLT by excess unlabeled angiotensin II provides the first direct demonstration of the presence of specific angiotensin II binding sites in the OVLT. The conjugate was shown to be biologically active by the elicitation of drinking behavior in all the animals used in this study. Therefore, the binding sites detected by the fluorescent tracer represent an angiotensin II-sensitive region of the brain involved in the mediation of central effects of angiotensin II. This technique offers an advance over other methods where testing of the biological activity and receptor binding cannot be done in the same animal. Quantification of the data may ultimately be possible. The results with this technique show that the OVLT has angiotensin II receptors accessible from the cerebrospinal fluid, since the route of administration of the conjugate was intraventricular. This finding supports the contention that we had made earlier that angiotensin II injected into the brain ventricles acts by activating receptors in the OVLT (*4*). The subfornical organ tissue in this experiment did not fluoresce but only zones 1 and 2 of the subfornical organ (*9*) were cut, and more dorsal areas have not been studied yet. High concentrations of conjugate were used to maximize receptor occupancy. Usually only 50 to 100 ng of angiotensin II in 1 μl of saline is used to produce reliable drinking (*1*), but we considered that this may be insufficient to occupy all the available receptors. An even higher dose of angiotensin II had to be used to inhibit the binding of conjugate, hence the nonphysiological amount injected.

Binding of angiotensin II in the brain had been established by binding of ^{125}I-labeled angiotensin II to cell membranes from dissected blocks of brain regions, but it is not certain that all of the binding sites are biological receptors. Our study shows that there are receptors in the OVLT that can be reached by the fluorescent conjugate in the brain ventricles.

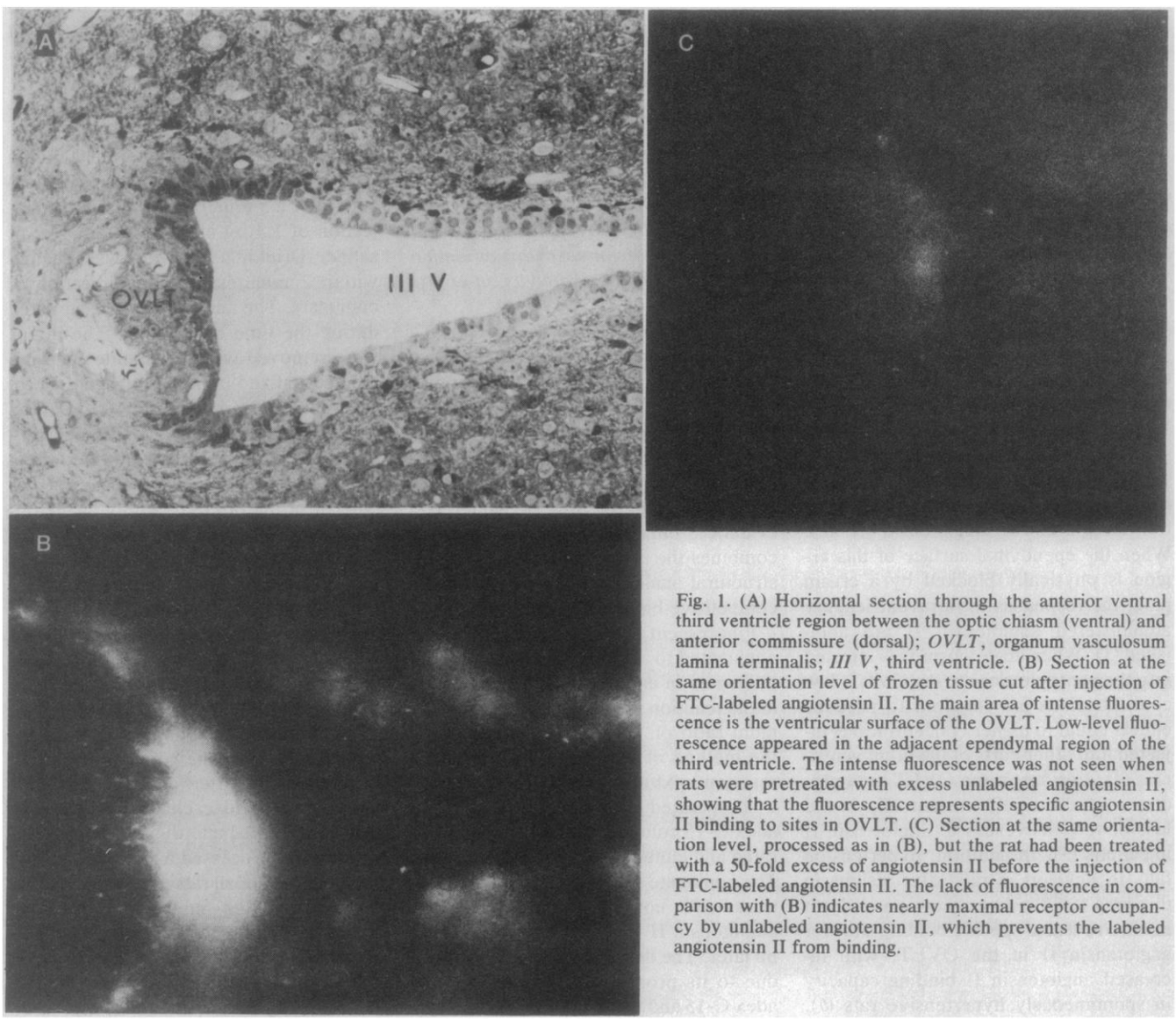

Fig. 1. (A) Horizontal section through the anterior ventral third ventricle region between the optic chiasm (ventral) and anterior commissure (dorsal); *OVLT*, organum vasculosum lamina terminalis; *III V*, third ventricle. (B) Section at the same orientation level of frozen tissue cut after injection of FTC-labeled angiotensin II. The main area of intense fluorescence is the ventricular surface of the OVLT. Low-level fluorescence appeared in the adjacent ependymal region of the third ventricle. The intense fluorescence was not seen when rats were pretreated with excess unlabeled angiotensin II, showing that the fluorescence represents specific angiotensin II binding to sites in OVLT. (C) Section at the same orientation level, processed as in (B), but the rat had been treated with a 50-fold excess of angiotensin II before the injection of FTC-labeled angiotensin II. The lack of fluorescence in comparison with (B) indicates nearly maximal receptor occupancy by unlabeled angiotensin II, which prevents the labeled angiotensin II from binding.

Histological localization by fluorescent hormone conjugate has been used to determine the histological localization of hormones in various nonneuronal tissue (*10*). In these earlier studies, however, competitive inhibition by excess unlabeled hormone was not included. Bioactive rhodamine-conjugated enkephalin was recently used in neuroblastoma cells to study the distribution of binding sites (*11*). The use of bioactive fluorescent derivatives of peptides in brain tissue can become a fast and accurate method for demonstrating the distribution of receptor regions in brain tissue.

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22 May 1980; revised 13 June 1980

Role of Ornithine Decarboxylase in Cardiac Growth and Hypertrophy

Abstract. *Inhibition of cardiac ornithine decarboxylase (ODC) by α-difluoromethylornithine (DFMO) did not prevent normal cardiac growth in mature rats but attenuated isoproterenol-induced hypertrophy. Hypertrophy caused by triiodothyronine was not prevented by DFMO. There appear to be both ODC-dependent and ODC-independent processes contributing to the subcellular mechanisms associated with growth, which must be considered in the potential laboratory and clinical use of DFMO.*

Ornithine decarboxylase (ODC) (E.C. 4.1.1.17) catalyzes the first and probably rate-limiting step in the biosynthesis of the polyamines putrescine, spermidine, and spermine (*1*, *2*). Polyamines are thought to play regulatory roles in both nucleic acid and protein syntheses, and the ODC molecule itself may act as an initiating factor for RNA polymerase I (*3*). Studies with regenerating rat liver, tumor cells, and a variety of prokaryotic and eukaryotic systems have demonstrated that ODC activity and polyamine concentrations are highest during rapid growth, differentiation, or replication and decrease as these processes cease or as the number of growing or dividing cells declines (*3*, *4*). Although these observations imply a functional role for ODC and the polyamines in the regulation of cellular development, not until recently has direct proof of this hypothesis been obtainable; the development of α-difluoromethylornithine (DFMO) (RMI 71, 782), a potent, selective, enzyme-activated irreversible inhibitor of ODC (*5*), has made it possible to show that ODC activity is necessary if cell replication is to progress normally. Incubation with DFMO retards the multiplication of cells in culture, and the growth of tumors in vivo and in vitro is inhibited by the drug (*6*). During mammalian development, DFMO causes the arrest of embryogenesis (*7*).

It is unclear, however, whether ODC is obligatory in all growth processes or whether only replication is affected. One way of testing this hypothesis is to examine the effects of inhibition of ODC in tissues that grow by hypertrophy of preexisting cells rather than by replication, a circumstance that prevails in the mature rat heart (*8*). Cardiac hypertrophy can be produced by aortic constriction, sympathetic hyperactivity, hyperthyroidism, or chronic stress or exercise (*9*), and in each case the changes in heart weight are preceded by ODC stimulation. In our study, DFMO has been used to determine if ODC activity is necessary for the normal growth of the mature heart and whether stimulation of ODC is an obligatory step in sympathetically induced (with isoproterenol) or hormonally induced (with triiodothyronine) cardiac hypertrophy.

Male Sprague-Dawley rats (Zivic-Miller) with initial body weights of 160 to 180 g were housed two per cage and given unlimited amounts of food and water. Cardiac hypertrophy was produced by daily subcutaneous administration of *dl*-isoproterenol (ISO) (0.5 mg/kg) or 3,3′,5-triiodo-*l*-thyronine (T_3) (0.1 mg/kg) (both from Sigma Chemical Company). Rats were killed by decapitation, and the heart ODC activity was determined in the 26,000*g* supernatant of hypotonic tris homogenates by a modification of the method of Russell and Snyder (*2*, *10*); heart and body weights were recorded, and the ratio of heart weight to body weight (a more specific index of cardiac hypertrophy) was calculated. Results are reported as the means ± the standard errors of the means of six or more animals, with levels of significance calculated by the two-tailed *t*-test.

Subcutaneous administration of 200 mg/kg of DFMO resulted in a rapid and profound decline in cardiac ODC, and

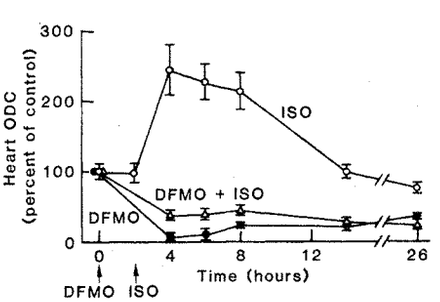

Fig. 1. Time course of cardiac ODC activity after a single treatment with DFMO or ISO, or both. Control ODC activity was 1.29 ± 0.14 nmole hour^{-1} g^{-1}.

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