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Wide distribution of immunoreactive renin in nerve cells of human brain

(aspartyl proteases/immunoperoxidase technique/angiotensin/brain peptides)

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ABSTRACT **By use of the indirect peroxidase–antiperoxidase complex immunocytochemical technique, antibody to purified human renal renin was applied to formalin-fixed paraffin sections of human cadaver brain. Immune reaction products were observed in most nerve cells in all areas of the brain examined; staining was limited to the soma and proximal dendrites. These experiments have confirmed the presence of a renin-like substance in central nervous tissue and suggest a more generalized function for "brain renin" than was previously anticipated.**

Controversy regarding the existence of a functional brain renin–angiotensin system prompted this investigation. Renin-like enzymatic activity has been demonstrated in low concentration in brain homogenates (1–3), and isolation of each of the components of the renin–angiotensin system from brain (4) suggested that the system might be functional there. Inability to distinguish renin enzymatic activity from that of cathepsin D, however, has generated much confusion (5–7). Hirose *et al.* (8) separated angiotensin I-generating activity from brain of nephrectomized rats into two peaks; one resembled cathepsin D, whereas the other was inhibited by antibody to purified hog renal renin. Such data, however, did not provide information about localization at the cellular level, whether the activity was in parenchymal or vascular components. Prompted by these findings, we sought to identify and localize renin in human brain by using immunocytochemical techniques and antibody elicited by homogeneous human renal renin (purified recently by one of us). Our studies have confirmed that a renin-like substance is present in human central nervous tissue with a distribution more widespread than previously anticipated.

MATERIALS AND METHODS

Human renal renin was purified by published methods, except that affinity chromatography was used as the final (seventh) step with purified immunoglobulin fraction of antisera raised against pure renin as the ligand (9). The product of elution represented a 500,000-fold purification and had a final specific activity of 1000 Goldblatt units/mg of protein. No contaminant bands were seen on $NaDodSO_4$/polyacrylamide or polyacrylamide disc-gel electrophoresis. The potency of immune rabbit antisera was determined by inhibition of the enzymatic activity of 0.5×10^{-4} Goldblatt unit of Haas human renal renin standard (no. 216, gift of Erwin Haas) compared to autologous preimmune serum and expressed as the dilution producing 50% inhibition—1:20,000 dilution for the antisera used in these experiments. Antibody diffusion in Ouchterlony plates (36 hr at room temperature) produced single precipitation lines with smooth fusion against pure (step 7) or partially pure (steps 6 and 5) renin. There was no precipitation with bovine spleen cathepsin D (Sigma), pure human hepatic cathepsin D (gift of Alan Barrett), or hog stomach pepsin (Sigma). The antibodies (1:10 dilution) did not inhibit the activity of partially purified human renal cathepsin D, as measured by the hemoglobin assay of Anson at pH 5.4 (10). They (1:20 dilution) did not inhibit the ability of cathepsin D or B, both partially purified from human kidney, to generate angiotensin I from human plasma at pH 5.5 (11).

Five-micrometer sections of formalin-fixed, paraffin-embedded human brain from six subjects (age range: 3 months–68 years), obtained between 3 and 18 hr after death, were processed by indirect peroxidase–antiperoxidase complex immunohistochemical methods (12, 13) by using as the first reactant rabbit antisera against human renal renin at dilutions of 1:1000–1:10,000 for 30 min–16 hr. Specificity controls included replacement of the primary antiserum by autologous preimmune rabbit serum at the same dilution and preincubation at a 1:2000 or 1:4000 dilution of the antiserum with 0.1–10 μg of purified, enzymatically active, human renal renin per ml for 12 hr.

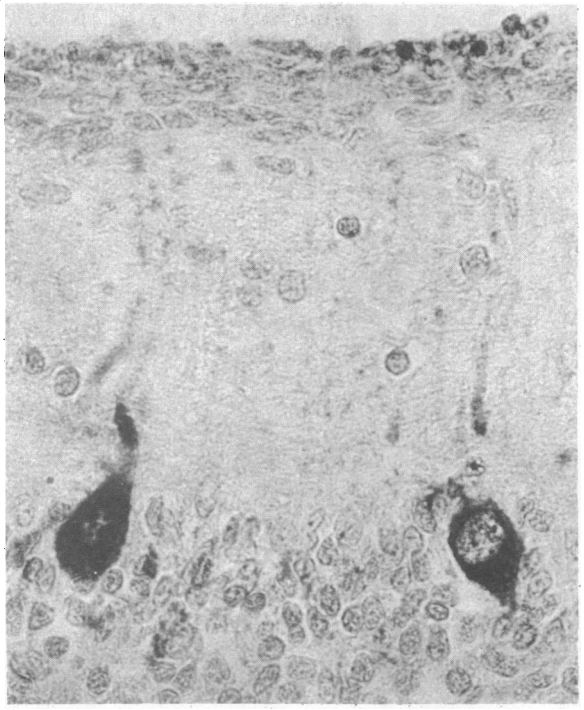

FIG. 1. Cerebellar cortex of a 3-month-old infant with normally persistent embryonic external granule cell layer (top). The large Purkinje neurons are intensely immunoreactive. (Immunoperoxidase technique with rabbit anti-human renal renin, 1:2000 dilution, for 45 min; hematoxylin counterstain; ×500.)

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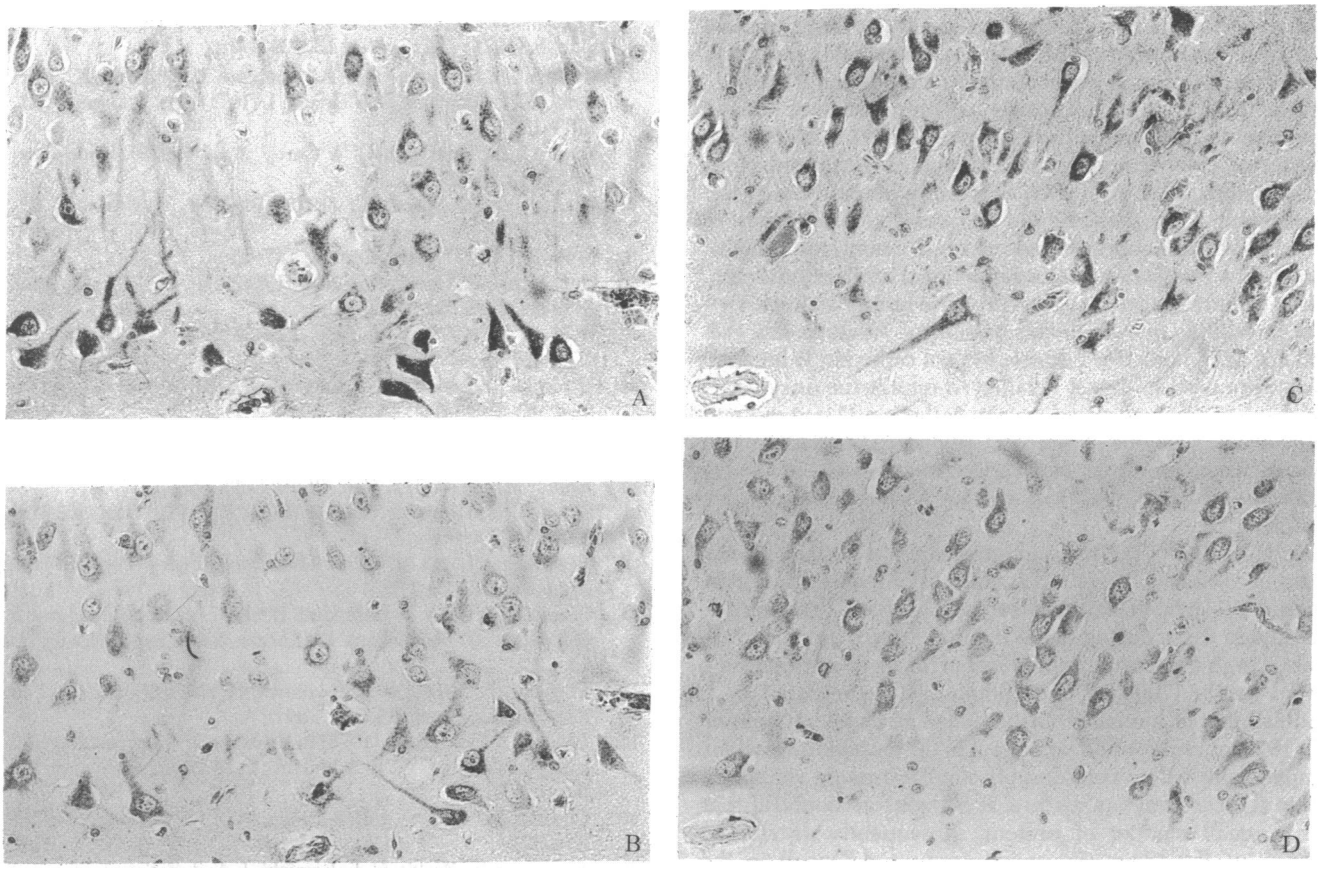

FIG. 2. Adjacent sections of the pyramidal layer of the hippocampus of an 8-year-old child (three blood vessel "markers" in lower right quadrant of *A* and *B*; one vessel "marker" in lower left corner of *C* and *D*) stained simultaneously by the immunoperoxidase method with rabbit anti-human renal renin (1:2000 dilution, for 45 min). In *B*, 1 ml of primary antiserum (1:2000 dilution) was preincubated overnight with 0.1 μg of purified human renal renin. After absorption, strongly immunoreactive nerve cells in *A* were less reactive in *B*, whereas moderately reactive cells failed to stain at all. The preparation of *C* and *D* was similar to that of *A* and *B*, respectively, except that 5 μg of antigen was used in *D*. After absorption with the higher concentration of antigen, staining was completely blocked. (Hematoxylin counterstain; ×150.)

RESULTS

Products of immune reaction were observed within nerve cells in all areas of the brain examined: cerebral neocortex (all laminae), hippocampus (dentate and pyramidal layers), amygdala, all basal ganglia, all thalamic and hypothalamic nuclei, cerebellar cortex (Purkinje and Golgi II neurons) and dentate nucleus, all levels of the brain stem, and spinal cord. All neurons were immunoreactive in some structures (inferior olive), roughly half were immunoreactive in many others, and only a few were occasionally immunoreactive in spinal gray matter. The reactive products were often uniformly granular. They were not compartmentalized in the cytosol and usually extended into proximal dendrites (Fig. 1). They were not encountered in fiber tracts nor did they form patterns suggestive of axonal arborizations. Scattered astrocytes were also positive. The ependyma did not stain. Other positive cells included some pinealocytes, neurohypophysial pituicytes, choroid epithelium, astrocyte-like cells in the organum vasculosum of the lamina terminalis, and many anterior pituitary cells. Staining of the above cells was absent when preimmune serum replaced the primary antiserum or when primary antiserum was preincubated with purified human renal renin; complete inhibition of staining occurred with 5 or 10 μg of renin per ml of dilute antiserum, and partial inhibition occurred with much smaller amounts (Fig. 2). Variable staining of blood vessel walls was indistinguishable from the nonspecific staining of connective tissue commonly seen in the immunoperoxidase method.

DISCUSSION

Our data demonstrate that most nerve cells throughout the human central nervous system contain a substance in the soma and proximal dendrites that possesses an antigenic determinant common to renal renin. The discrepancy between the widespread reactivity found by immunocytochemical methods and the low yield of renin enzymatic activity extracted from homogenized tissue (5) can be explained either by concomitant extraction of inactivating proteases or by the substance's presence in an inactive form (14). Indeed, the large amount of reactivity found intracellularly would argue for the presence of a protease in inactive form.

The distribution of renin-reactive nerve cells is much more general than that of known neuronal systems containing peptides or biogenic amines with neurotransmitter functions (15). Our findings correlate with the wide distribution for the components of the renin–angiotensin system suggested by their isolation from various brain regions in several species: angiotensinogen in rats (16, 17), renin in hogs (14), and angiotensin-converting enzyme in humans, dogs, guinea pigs, and rabbits (18–20). They contrast with the more localized distribution for angiotensin II demonstrable by the immunoperoxidase technique in perikaryons of the magnocellular nuclei of rat hypothalamus (21). This focal distribution is probably more relevant to the observed effects of the renin–angiotensin system elicited by injection of its components into cerebral ventricles (increased drinking, release of vasopressin and corticotropin, and elevation of blood pressure) (21, 22).

The substance we have demonstrated may be renin, but alternative explanations must also be considered. The possibility that the renal immunogen contained a contaminant coincidental in brain is unlikely because our preparation of homogeneous human renal renin has the highest specific activity thus far reported and because minute amounts of the immunogen blocked staining after incubation with the antiserum. Somewhat more tenable is the possibility of crossreactivity of antirenin antibody with an homologous sequence in a nonrenin protein in brain. The close structural and functional similarity of renin to other members of the aspartyl protease family makes it virtually impossible to exclude this possibility. Nevertheless, the substance herein described is probably not cathepsin D because the antisera used to detect it failed to inhibit the enzymatic activity of partially purified human cathepsin D and did not crossreact with bovine or human cathepsin D in double immunodiffusion. Moreover, its distribution is unlike the known localization for cathepsin D in lysosomes, which are absent from dendrites (23).

The possibility of postmortem intracellular diffusion of renin cannot be excluded. Nevertheless, this would require that renin (molecular weight 40,000) pass through the blood–brain barrier and remain there during tissue fixation. Moreover, if one assumes a high circulating renin level of 25 ng of angiotensin I per ml per hr (7000 ng of angiotensin I is generated by 1 Goldblatt unit of human renin), a total of 10 Goldblatt units or only 10 μg of renin would be present in the entire intravascular compartment with a considerably smaller amount contained within the cerebral vasculature. It is therefore doubtful that such a small amount of protein, subsequently distributed throughout the brain and spinal cord, could result in the widespread reactivity demonstrated by our experiments.

The relationship of the neuronal immunoreactivity herein described to renal renin must be established by isolation, complete purification, characterization, and substrate specificity studies. If this immunoreactivity is due to a protein with renin-like biological activity, then more generalized functions for "brain renin" must be anticipated. Such functions might involve the modulation of other peptidergic systems. Alternatively, by analogy to the known effects of renin on renal glomerular blood flow (24, 25), brain renin might be active in the microautoregulation of regional cerebral blood flow.

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