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Bradykinin-like immunoreactive neuronal systems localized histochemically in rat brain

(kinin distribution/indirect immunofluorescence/central nervous system)

FERNANDO M. A. CORRÊA*, ROBERT B. INNIS, GEORGE R. UHL, AND SOLOMON H. SNYDER†

Departments of Pharmacology and Experimental Therapeutics and Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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ABSTRACT **Bradykinin-like immunoreactive structures were localized in rat brain by the indirect immunofluorescence method. Specificity of staining was demonstrated by: (*i*) the absence of fluorescence when preimmune serum was used, (*ii*) the disappearance of fluorescence when sera were preadsorbed with bradykinin, and (*iii*) the presence of identical staining with two different antisera. Immunoreactive neuronal cells are observed only in the hypothalamus, with especially dense clusters overlying the periventricular and dorsomedial nuclei. Fibers and varicose processes are observed in the periaqueductal gray matter, hypothalamus, perirhinal and cingulate cortices, the ventral portion of caudate-putamen, and the lateral septal area.**

Bradykinin, a nonapeptide first found in mammalian blood (1), is involved in the mediation of several pathophysiological conditions, including inflammation, pain generation, cardiovascular shock, and hypertension (2–4). In addition, a variety of evidence suggests a role for a bradykinin-like system in mammalian brain. Specific bioassay and radioimmunoassay with potent and selective antisera indicate the presence of bradykinin-like activity in extracts of brain tissue (refs. 5–7; unpublished data). Moreover, direct administration of bradykinin in doses from 10 fmol to 1 pmol into the lateral ventricles or restricted areas of the brain produces selective autonomic and behavioral responses (8–10).

In the present study, by using two specific antisera to bradykinin, we describe neuronal systems containing bradykinin-like immunoreactivity.

MATERIALS AND METHODS

Immunofluorescence. Bradykinin-like immunoreactivity was visualized by the indirect immunohistofluorescence method of Coons and coworkers (11), as described earlier (12). Sprague–Dawley male rats (100 g) were injected intracerebroventricularly with 50 μg of colchicine (Sigma) dissolved in 20 μl of 0.9% NaCl 48 hr before they were killed. Animals were perfused through the heart first for 10 sec with ice-cold normal saline and then for 5–10 min with ice-cold phosphate-buffered 4% depolymerized paraformaldehyde solution. Brains were postfixed for 90 min in this perfusate and then soaked for at least 24 hr in 7% sucrose/0.6 M phosphate buffer, pH 7.4. Brain slabs approximately 0.5 mm thick were rapidly frozen onto cryostat chucks with liquid nitrogen. Sixteen-micrometer sections were cut at −20°C in a Harris cryostat and thaw-mounted onto slides coated with gelatin/chrome alum. Sections were stained at 37°C for 30 min with primary antisera diluted 1:25 with phosphate-buffered saline containing 0.2% Triton X-100. After three 5-min washes with phosphate-buffered saline/0.05% Triton, the sections were exposed for 15 min at 37°C to fluorescein-conjugated guinea pig antibody against rabbit IgG (Cappel Laboratories, Cochranville, PA) diluted 1:40 with phosphate-buffered saline/0.1% Triton. The sections were then washed three times (5 min each) in phosphate-buffered saline/0.2% Triton, dipped in H_2O, and mounted with 0.5 M sodium bicarbonate buffer (pH 8.4) diluted with an equal volume of glycerol. Each section was examined under darkfield conditions with a Zeiss Universal fluorescence microscope by two independent observers. Each level described in detail here was examined in at least three brains. Adjacent sections from each brain were stained with preimmune sera and with control sera, preadsorbed overnight at 4°C with 15 μM bradykinin (Bachem Inc., Marina del Rey, CA). In all descriptions of immunoreactivity, we refer only to immunofluorescence that was essentially eliminated by this preadsorption. Adjacent sections were stained with cresyl violet, and the drawings of Konig and Klippel (13) and Palkovitz and Jacobowitz (14) were consulted for anatomic localization.

Antisera Preparation. Antisera were raised in rabbits to bradykinin coupled to hemocyanin with glutaraldehyde. Hemocyanin (2.5 mg) was mixed with 5 mg of bradykinin and suspended in 0.5 ml of 0.2 M sodium acetate buffer (pH 5). Ten microliters of ^{125}I-labeled [Tyr^8]bradykinin solution (50,000 cpm) was added to evaluate the percentage coupling of bradykinin to the carrier. Seventy microliters of 2.5% glutaraldehyde was added, and the reaction was carried out at 25°C for 6 hr with agitation. After centrifugation and washing, the percent coupling of bradykinin was 15%. The final pellet was resuspended in 1 ml of saline. Five rabbits were injected each with an initial dose of 100 μl of suspension in 2 ml of complete Freund's adjuvant. Animals were bled every 30 days and injected with a 50-μl boost suspended in 2 ml of incomplete Freund's adjuvant. All animals developed antibodies against bradykinin. However, only two of five developed a titer higher than 1:400, and sera from these two animals were used in our present experiments.

RESULTS

Characterization of Bradykinin Antisera. The antiserum (G1) used routinely for immunohistochemical localization has a high titer in the radioimmunoassay. At 1:12,800 dilution, 30% of added ^{125}I-labeled [Tyr^8]bradykinin is bound to the antiserum, while at 1:3200 dilution, 50% is bound (Fig. 1). The radioimmunoassay is sensitive: 50% displacement of ^{125}I-labeled [Tyr^8]bradykinin binding to the antisera is apparent at 1 nM unlabeled bradykinin (Fig. 1). The slope of the displacement

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* Permanent address: Department of Pharmacology, School of Medicine, Ribeirão Preto–USP, Riberão Preto, Sao Paulo, 14100, Brazil.

† To whom all correspondence should be addressed.

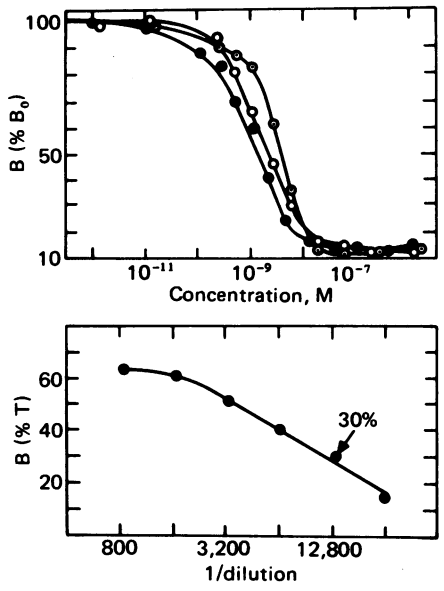

FIG. 1. Properties of brandykinin antiserum G1. (*Upper*) Displacement of ^{125}I-labeled [Tyr^8]bradykinin from antisera raised in rabbits to bradykinin. The antiserum was used at a dilution of 1:12,800. Free and bound ligand were separated by dextran-coated charcoal, and bound (B) is expressed as percent of total ligand bound (B_0) in the absence of displacer. Displacement is given for bradykinin (●), lysyl-bradykinin (○), and methionyllysyl-bradykinin (⊙). (*Lower*) Titration curve of the same antisera. Bound (B) is expressed as percent of total radioactive ligand (T) added to the assay. Thirty percent of ^{125}I-labeled [Tyr^8]bradykinin is bound to antibody at an antiserum dilution of 1:12,800.

curve is steep, and between 10 and 90% displacement of added ^{125}I-labeled [Tyr^8]bradykinin occurs over the concentration range of 0.1–10 nM unlabeled bradykinin (Fig. 1). The antiserum appears highly specific. Lysyl-bradykinin and methionyllysyl-bradykinin are 20–50% as potent as bradykinin itself in competing for ^{125}I-labeled [Tyr^8]bradykinin binding. By contrast, a large number of other peptides have no effect on ^{125}I-labeled [Tyr^8]bradykinin binding to the antiserum when tested at concentrations as high as 10 μM. Peptides inactive at 10 μM include glycylglycine, substance P, neurotensin, [Met]-enkephalin, [Leu]enkephalin, angiotensin I, angiotensin II, prolylleucyltyrosine, prolylleucylphenylalanine, secretin, and prolactin.

The second antiserum (G2) is used in radioimmunoassay at a dilution of 1:3000, where 25% of added ^{125}I-labeled [Tyr^8]-bradykinin is bound. Compared to antiserum G1, antiserum G2 has a higher specificity for bradykinin relative to its physiologic precursors, as lysyl-bradykinin and methionyllysyl-bradykinin are only 10–20% as potent as bradykinin itself in competing for ^{125}I-labeled [Tyr^8]bradykinin binding. For fluorescence staining, this antiserum was used at a dilution of 1:10, as opposed to 1:25 for the routine antiserum G1. Although antiserum G2 must be used at a different titer and shows different relative affinities for bradykinin-related peptides, we obtain fluorescence patterns in all areas of the rat brain identical to those of our routinely used antiserum G1.

General Characteristics of Fluorescent Staining of Immunoreactive Bradykinin. In all parts of the central nervous system where immunoreactive bradykinin can be detected, it is localized to neuronal cells and fibers. To determine whether the immunoreactivity represents bradykinin or closely related structures, we have performed preadsorption experiments with bradykinin and other peptides. Control experiments using preimmune sera or sera previously incubated with 15 μM bradykinin show negligible fluroescence. By contrast, preadsorption with hemocyanin (20 mg/ml), neurotensin (100 μM), cholecystokinin octapeptide (100 μM), pentagastrin (100 μM), angiotensin II (100 μM), [Leu]enkephalin (100 μM), or secretin (100 μM) fails to eliminate the bradykinin-like fluorescence. Since lysyl- and methionyllysyl-bradykinin also react with the antisera in radioimmunoassay, we cannot rule out the possibility that observed histofluorescence is due to one of these or other related kinins. Hereafter, what is observed histochemically as bradykinin-like immunoreactivity will be referred to simply as "bradykinin."

Bradykinin-containing cells vary considerably in shape, from round to fusiform. The fluorescence is granular, localized over the cytoplasm and not over the nucleus, and often extends in processes for long distances. In some cases, single large processes are apparent while other cells appear to produce large numbers of processes. These cells tend to be large, with a mean diameter of about 20 μm. Fibers located close to the cells tend to be fine, with few varicosities. Other terminal fibers are thicker with larger, regularly spaced varicosities (Fig. 2).

While most of the experiments have used colchicine-pretreated rats, some experiments have utilized untreated animals. In these latter animals, fibers appear quite similar to those in colchicine-pretreated animals but the fluorescence of neuronal cell bodies is substantially diminished.

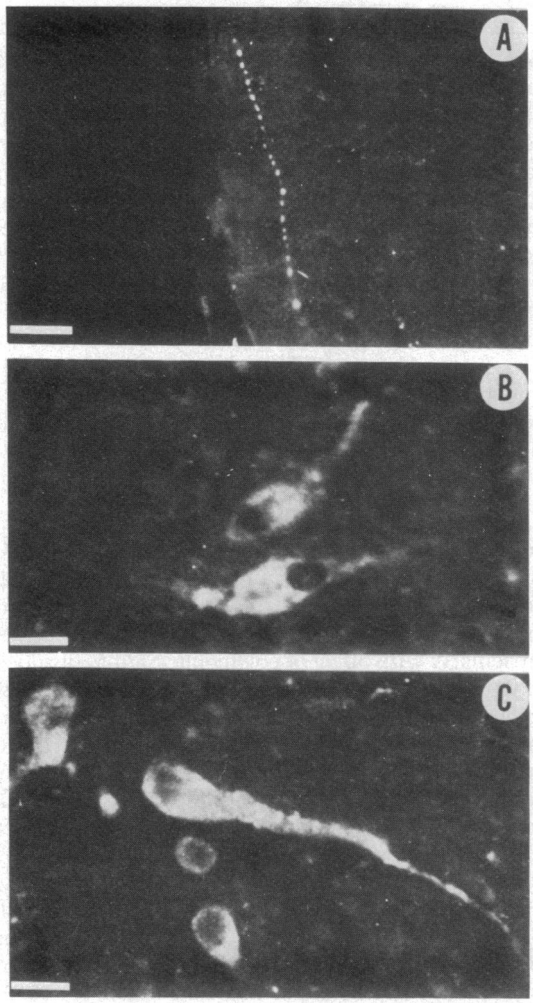

FIG. 2. Immunofluorescence micrographs of bradykinin. (*A*) A varicose fiber in layer I of perirhinal cortex. The varicosities are large and prominent with short intervaricose segments. (*B*) Two positively stained perikarya from lateral hypothalamus. (*C*) A positively stained neuron in the lateral hypothalamus with fluorescence extending into a wide and long process. Bars = 20 μm.

Distribution of Immunoreactive Bradykinin at Various Levels of the Rat Central Nervous System. Careful examination of the spinal cord at mid-cervical and upper-cervical regions fails to reveal any bradykinin-like immunoreactivity. No bradykinin is observed in the medulla oblongata, though serial sections have been examined at 200-μm intervals. In the pons and mesencephalon over the entire length of the periaqueductal gray matter, sparse numbers of fibers are observed oriented radially to the aqueduct, while no fluorescent cells are evident.

The only bradykinin-containing cells observed throughout the central nervous system are contained in hypothalamic areas. In the posterior hypothalamus, a small number of cells are observed in the most dorsal portion of the hypothalamus, medial to the fields of Forel, while few fluorescent fibers are noted. In the medial hypothalamus, the most dense collection of cells is noted in the dorsal portion, extending from the midline to the most lateral extent of the hypothalamus. These cells overlie the periventricular hypothalamus, the ventral portion of the dorsomedial hypothalamus, and the lateral hypothalamus including the medial forebrain bundle. A dense number of bradykinin-containing fibers oriented in the ventrodorsal di-

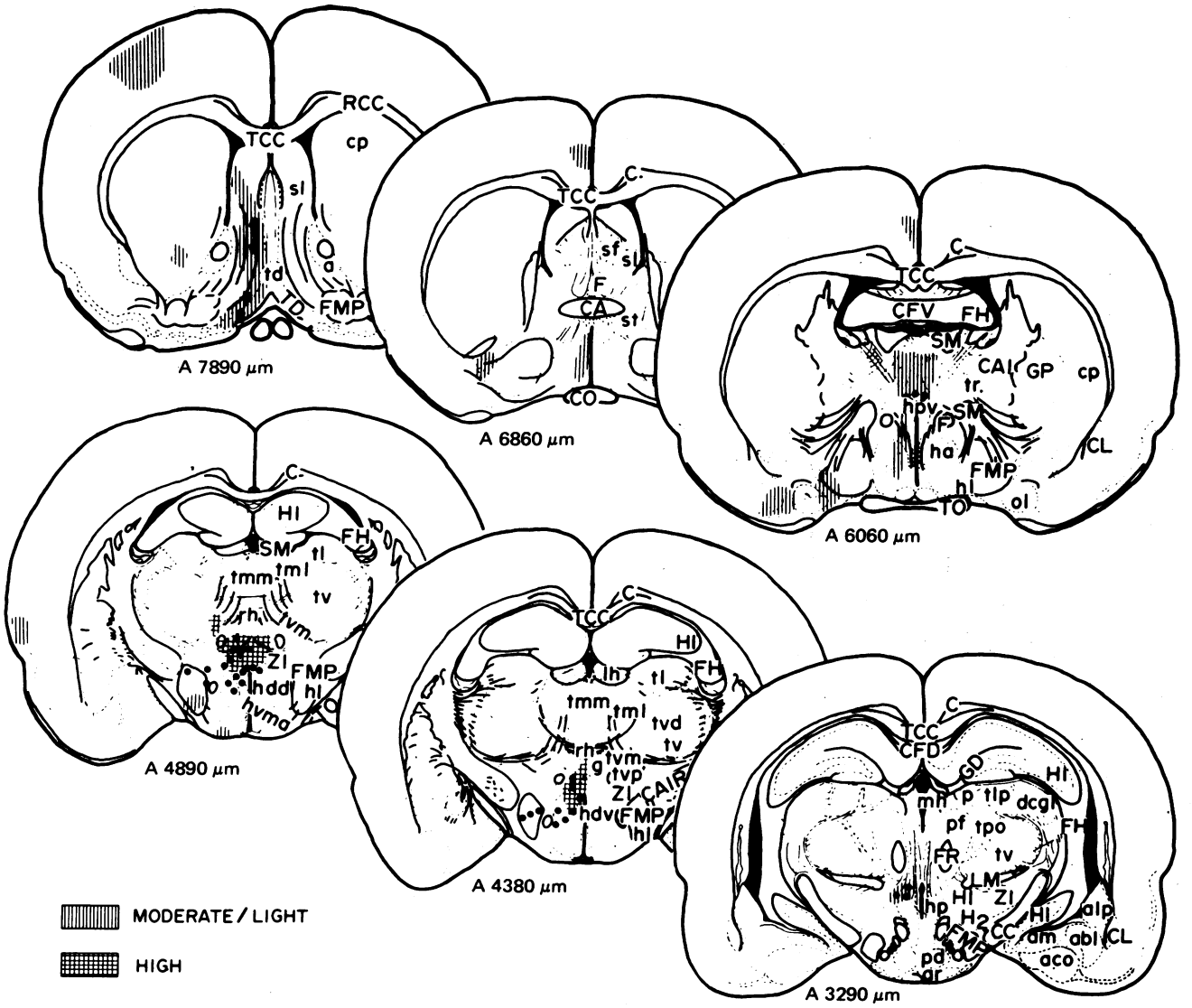

FIG. 3. Distribution of bradykinin immunofluorescence. Black dots stand for cell bodies. Densities of fibers are graded moderate/light and heavy. Abbreviations: C, cingulum; CA, commissura anterior; CAI, capsula interna; CAIR, capsula interna, pars retrolenticularis; CC, crus cerebri; CFD, commissura fornicis dorsalis; CFV, commissura fornicis ventralis; CL, claustrum; CO, chiasma opticum; F, columna fornicis; FH, fimbria hippocampi; FMP, fasciculus medialis prosencephali; FR, fasciculus retroflexus; GD, gyrus dentatus; GP, globus pallidus; HI, hippocampus; H_1 H_2, Forel's fields; LM, lemniscus medialis; RCC, radiato corporis callosi; SM, stria medullaris thalami; TCC, truncus corporis callosi; TD, tractus diagonalis (Broca); TO, tractus opticus; ZI, zona incerta; a, nucleus accumbens; abl, nucleus amygdaloideus basalis, pars lateralis; aco, nucleus amygdaloideus corticalis; alp, nucleus amygdaloideus lateralis, pars posterior; am, nucleus amygdaloideus medialis; ar, nucleus arcuatus; cp, nucleus caudatus putamen; dcgl, nucleus dorsalis corporis geniculati lateralis; g, nucleus gelatinosus; ha, nucleus anterior hypothalami; hdd, nucleus dorsomedialis hypothalami, pars dorsalis; hdv, nucleus dorsomedialis hypothalami, pars ventralis; hl, nucleus lateralis hypothalami; hp, nucleus posterior hypothalami; hpv, nucleus periventricularis hypothalami; hvma, nucleus ventromedialis hypothalami, pars anterior; lh, nucleus habenulae lateralis; mh, nucleus medialis habenulae; ol, nucleus tractus olfactorii lateralis; p, nucleus pretectalis; pd, nucleus premamillaris dorsalis; pf, nucleus parafascicularis; rh, nucleus rhomboideus; sf, nucleus septalis fimbrialis; sl, nucleus septi lateralis; st, nucleus interstitialis striae terminalis; td, nucleus tractus diagonalis (Broca); tl, nucleus lateralis thalami; tlp, nucleus lateralis thalami, pars posterior; tml, nucleus medialis thalami, pars lateralis; tmm, nucleus medialis thalami, pars medialis; tpo, nucleus posterior thalami; tr, nucleus reticularis thalami; tv, nucleus ventralis thalami; tvd, nucleus ventralis thalami, pars dorsomedialis; tvm, magnocellular part of ventral thalamic nucleus; tvp, nucleus ventralis medialis thalami, pars parvocellularis. Levels are from ref. 13.

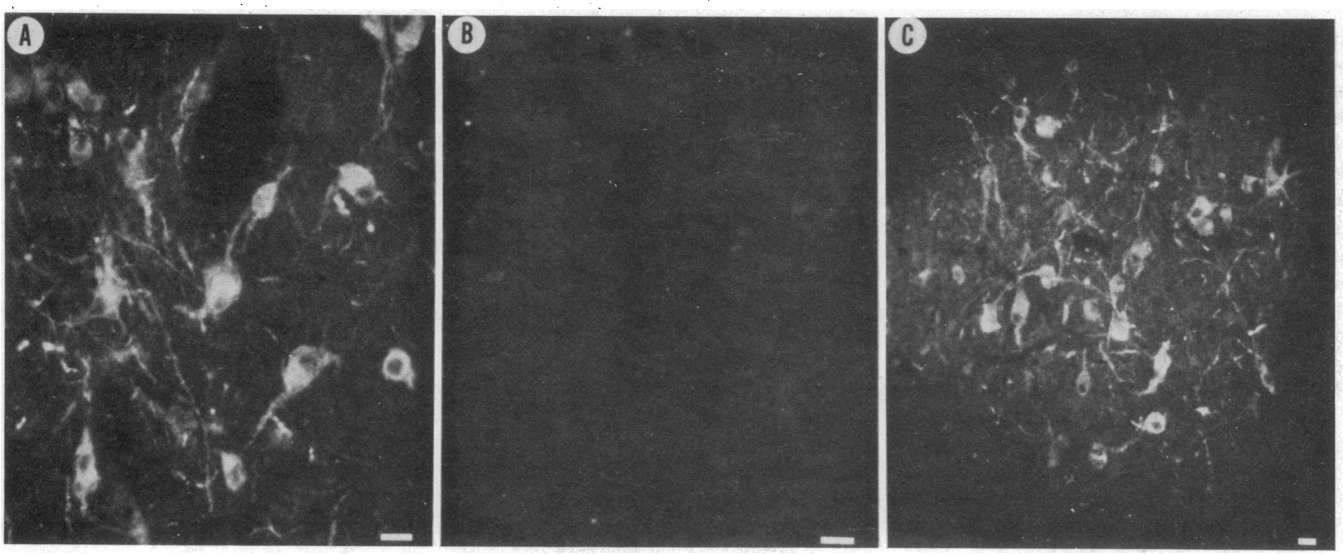

FIG. 4. Immunofluorescence micrographs of bradykinin fibers and cell bodies in the dorsomedial nucleus of the hypothalamus. (*A*) Immunofluorescence located in the cytoplasm of cells and extending in fibers from the perikarya. (*B*) The almost complete elimination of fluorescence when the primary antisera has been preadsorbed overnight with 15 μM bradykinin. (*C*) Bradykinin-positive cells and fibers just lateral to the dorsomedial nucleus of the hypothalamus. Bars = 20 μm.

rection seems to emerge just dorsal to the bradykinin cells in the midline (Figs. 3 and 4). These cells do not appear grouped into distinct clusters, but are spread throughout the dorsal portion of the hypothalamus.

At a more anterior level of the medial hypothalamus (A 4900 μm) a large number of cells is also observed, but appears to be located more dorsally (Fig. 3). In the midline, a dense cluster of cells lies just dorsal to the third ventricle. Cells also occur somewhat more dorsally overlying the nucleus reuniens and zona incerta as well as more laterally overlying the dorsal portion of the dorsomedial hypothalamus and the lateral hypothalamus including the medial forebrain bundle. A high density of fibers again appears to emerge dorsally from the midline-located bradykinin cells. Sparse numbers of bradykinin fibers are localized in the ventral portion of the medial forebrain bundle. Also at this level a limited number of bradykinin fibers are observed in superficial layers of the cerebral cortex just dorsal to the rhinal sulcus.

Fewer bradykinin cells are observed in the anterior hypothalamus (Fig. 3). These are localized in the midline at the dorsal border of the stellatocellularis periventricular nucleus. Sparse fibers are localized dorsally to these cells overlying the nucleus paratenialis. Low densities of fibers pass dorsoventrally over the medial portion of the anterior hypothalamus. A few groups of fibers are also observed over the ventrolateral border of the medial forebrain bundle, the pyriform cortex, and also the cingulate gyrus. More anteriorly, fibers are noted close to the midline of the preoptic area, as well as over the lateral portion of the medial forebrain bundle and the most ventral portion of the globus pallidus.

At precommissural levels (Fig. 3), fibers of low density pass ventrodorsally throughout the lateral septal area. A few fibers are also observed in the ventral portion of the caudate-putamen. At this level, sparse fibers are also observed in the frontal cortex about 2 mm lateral to the midline.

DISCUSSION

In immunohistochemical studies, a major task is to determine whether the observed immunoreactivity in fact represents the substance toward which the antisera were raised. For bradykinin, our antisera also recognized the physiological bradykinin precursors, lysyl- and methionyllysyl-bradykinin. However, its specificity is evident in its failure to detect a wide variety of other peptides that do not contain the bradykinin nonapeptide sequence. The antiserum used in the present study has a substantially higher titer for bradykinin in radioimmunoassay than those developed in the past (15), a factor that also favors specificity. Though the antiserum is used in higher concentration for immunohistochemical experiments than for radioimmunoassay, the preadsorption controls support its selectivity for bradykinin or closely related kinins.

Thus, support of the specificity of the fluorescence labeling derives from the absence of staining when preimmune sera are used and from the disappearance of staining after preadsorption with bradykinin. Further evidence comes from our finding that a second antiserum with different recognition patterns gives identical fluorescence staining. This result strongly supports the conclusion that the fluorescence is due to a substance either identical or closely similar to the bradykinin nonapeptide. Nevertheless, one cannot completely rule out crossreactivity with other antigens.

In a limited number of studies, biochemical evidence for endogenous bradykinin-like material in brains of rats and other mammalian species has been obtained. Most studies demonstrating endogenous bradykinin-like activity in brain have used bioassays, demonstrating the presence in brain extracts of material whose pattern of effects on various smooth muscles is unique to bradykinin and closely related kinins (5–7, 16). These effects include relaxation of the rat duodenum, contraction of the estrous rat uterus and guinea pig ileum, and lowering of blood pressure when administered systemically. These effects are potentiated selectively by the "bradykinin potentiating factor" from *Bothrops jararaca* snake venom and, like bradykinin, are resistant to the effects of trypsin but readily degraded by α-chymotrypsin. Preliminary radioimmunoassay studies indicate the presence of endogenous immunoreactive bradykinin in rat brain (unpublished data).

One can speculate as to the possible functions of the bradykinin neuronal systems identified in the present study. Positive correlations can be established between the anatomical distribution of the immunoreactivity presently observed and the proposed sites of action of centrally injected bradykinin. In-

jections of bradykinin into rat lateral ventricle raise blood pressure, an effect that is abolished by lateral septal lesions and reproduced by injections directly into this area (8, 9, 17). Fibers presently observed in the lateral septal area could constitute the anatomical substrate for these cardiovascular effects. The region of the periaqueductal gray has been associated with analgesia (18). Injection of bradykinin close to the periaqueductal gray elicits analgesia (19, 20), which could relate to the bradykinin fibers visualized in this region. Injections of small doses of bradykinin in the medial and anterior hypothalamus selectively elicit hyperthermia (unpublished observation), which could indicate a thermoregulatory role for the abundant bradykinin cells and fibers in this area.

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